Division of

Cancer Biology and Diagnosis

1986 Annual Report
Volume I

October 1, 1985-
September 30, 1986

U.S. DEPARTMENT
OF HEALTH
AND HUMAN SERVICES

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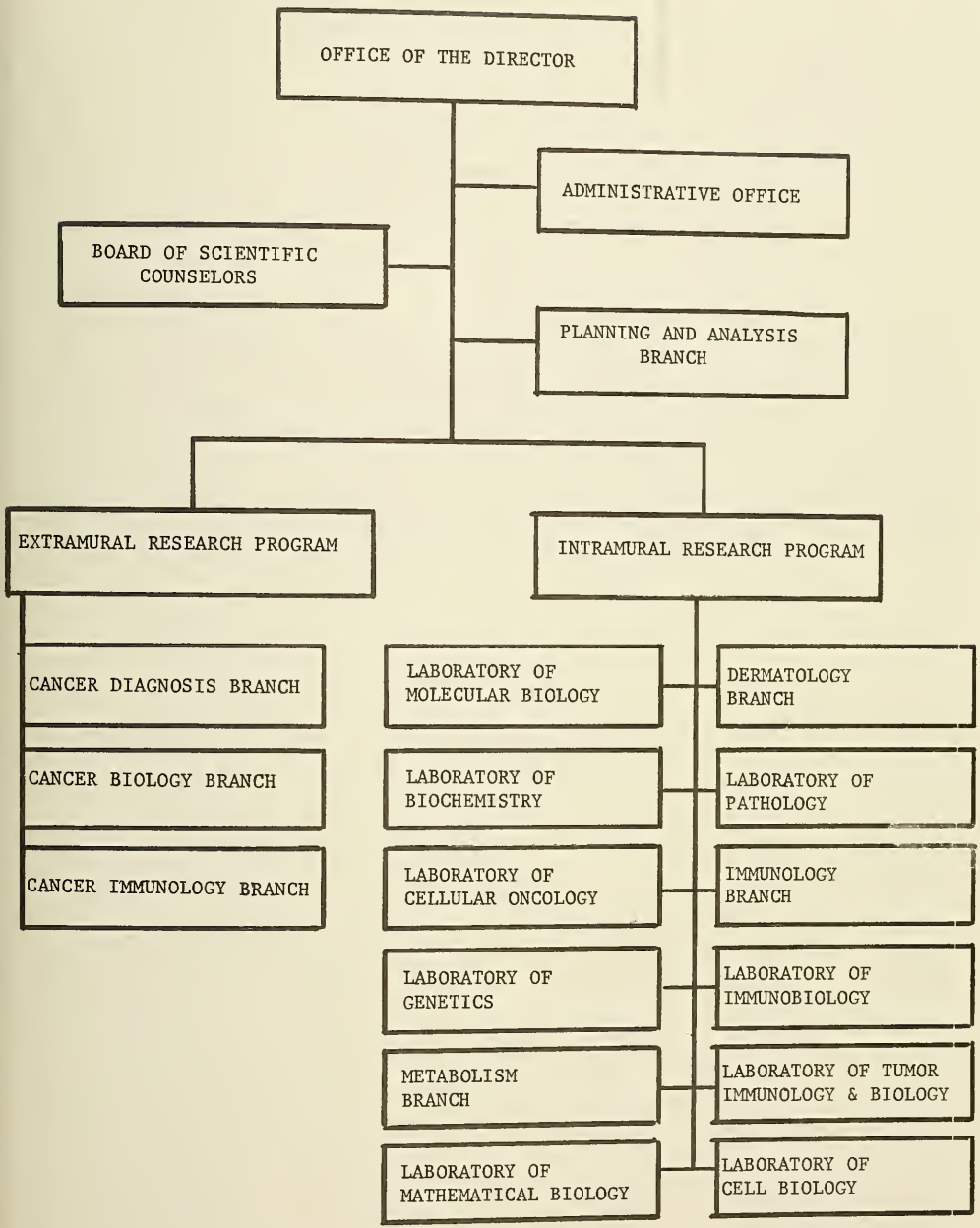
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DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE

INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1985 through September 30, 1986

INTRODUCTION

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

The research program of the Laboratory of Molecular Biology (Dr. Ira Pastan, Chief), focuses on the study of the factors that control gene expression. The goal is to understand how genes work, and to use this information to manipulate gene expression to prevent or cure disease. Major emphasis is placed on investigations of the molecular basis for the development of multidrug resistance. A human gene encoding multidrug resistance (mdr-1) has been isolated, and mdr-1 expression has been measured in normal and neoplastic human tissues. High levels of expression have been found in pheochromocytomas and normal human adrenals, colon carcinomas and normal colon, and a few cancers resistant to drug treatment. This suggests that measurement of mdr-1 RNA in cancers may be useful in designing treatments for different types of cancer. The product of the mdr-1 gene is a 170 kd membrane glycoprotein that binds vinblastine and related drugs and functions to keep the intracellular concentration of these anticancer drugs low. Other drugs can overcome drug resistance by preventing the anticancer drugs from binding to the membrane glycoprotein encoded by mdr-1. A sophisticated fluorescence technique has been developed which makes it possible to evaluate drugs that interfere with this efflux, and thus might be useful as adjunctive agents for chemotherapy.

In another research project, the epidermal growth factor receptor gene and its promoter region have been cloned. This promoter region resembles the promoters of SV40 virus and the human H-*ras* gene, suggesting that expression of certain growth control genes may be regulated by a common mechanism. These studies should further our understanding of how oncogenes regulate cell growth. Fibroblasts transformed by oncogenes (such as v-*mos* or v-*ras*) show an inhibition of type I collagen and fibronectin synthesis and their transcripts. This system is used to study how such oncogenes disrupt the differentiation program of cells.

The mechanism of the regulation of the growth of cancer cells has been studied using a number of different biochemical and genetic systems. Cancer cell growth can be inhibited by cAMP. cAMP-resistant Chinese hamster ovary cell mutants with altered cAMP dependent protein kinases have been isolated and this cell culture system has been utilized to study the mechanism by which cAMP regulates cell growth and gene expression. cAMP dependent expression of certain eukaryotic promoters has also been shown to depend on the presence of an intact cAMP dependent protein kinase system. In other studies using temperature-sensitive mutants with an altered beta-tubulin, the gene encoding a mutant beta-tubulin has been identified. The major excreted protein (MEP) of transformed mouse cells has been shown to be an acid-activable thiol protease. Levels of synthesis and secretion of this protease are regulated at the level of transcription by many different oncogenes, tumor promoters, and several growth factors.

A DNA-mediated gene transfer system has been developed and is being utilized to study growth regulation in mammalian cells. Molecular cloning of growth inhibitory sequences is being pursued and the action(s) of these growth inhibitory sequences, and those of growth stimulatory genes, is being investigated using a new magnetic affinity cell sorting method. Applications of this method, in particular for the introduction of growth inhibitory and/or growth stimulatory genes, the anti-sense complement of these genes, or regulatory sequences flanking these genes, are being explored.

Regulation of the *gal* operation of *E. coli* is being studied. *gal* is repressed by two repressor molecules binding at two operators, one on either side of the promotor region. Evidence suggests that the two repressors interact to form a

DNA loop between them, resulting a conformation unfavorable for transcription. A model has been developed to enable the identification of the specific genetic factors involved in how the allosteric change in the cyclic AMP receptor protein of E. coli is brought about by cAMP. The identification and characterization of these factors is underway.

E. coli lon mutants are defective in cell division regulation after DNA damage. This defect is due to stabilization of a highly unstable cell division inhibitor, the product of the SulA gene. lon mutants also overproduce capsular polysaccharide. Three regulatory genes have been identified which participate in the regulation or capsule synthesis genes and the protein products of two positive regulatory loci have been identified. Studies on cells devoid of lon activity demonstrate the existence of other ATP-dependent proteolysis systems in E. coli. A genetic selection for mutants with increased activity for other proteases have been developed, by selecting for mutants resistant to SUl A.

Using a biochemical screen for the ATP-dependent degradation of casein, a new, multi-component ATP-dependent protease in cells devoid of lon activity has also been identified. This system provides a model for studying the role of protein degradation in regulatory cell growth control.

An in vitro system that replicates supercoiled plasmid DNA containing the λ origin of replication has been utilized to study the mechanism of DNA replication. The initiation process requires two phage initiation proteins O and P, and many E. coli proteins, RNA transcription in the region of the origin, and a specific site on the DNA. The minimal DNA site required for initiation has been determined. Two of the required E. coli proteins have been purified and are being characterized biochemically.

Progress has been made in the development of immunotoxins for the treatment of adult T-cell leukemia (ATL) and ovarian cancer. Two patients with ATL have received a conjugate of Pseudomonas exotoxin A (PE) with an antibody to the interleukin 2 receptor (anti-TAC). This study showed that small amounts of PE-anti-TAC could be given safely to patients. Animal studies demonstrated the effectiveness of using PE and ricin A chain containing immunotoxins against human ovarian cancer. Some of these immunotoxins are now being evaluated for possible clinical use. Monoclonal antibodies are being evaluated for use in constructing immunotoxins specific for human ovarian carcinomas. These studies have identified normal tissues that are reactive with antibodies to the human transferrin and IL-2 receptors, as well as with a series of monoclonal antibodies generated to human ovarian carcinoma cells.

The interactions of cells with fibronectin and collagen have provided a system for studying the complex mechanisms of cell adhesion and their relevance to cell migration and metastases. The regions of fibronectin required for binding to cells have been defined, and membrane-associated receptors for fibronectin and collagen have been characterized. Fibronectin binding and adhesion requires a 140K protein complex and a second protein of 45K. It has been shown that a major protein involved in binding collagen is a 47K phosphoprotein. Levels of this protein decrease after malignant transformation, but its phosphorylation is substantially elevated. This protein is a novel, major heat shock-regulated protein.

The investigations carried out in the Laboratory of Biochemistry (Dr. Maxine Singer, Chief), explore fundamental questions about the biology of organisms ranging from bacteria through yeast, invertebrates, and mammals including humans. Such studies illuminate not only normal biological processes but also the aberrant events associated with disease, and in particular cancer.

Previous studies led to the development of a model for the pathophysiology of scurvy in which the observed defects in collagen and proteoglycan synthesis are caused by changes in humoral factors during scurvy-induced fasting, rather than through the role of ascorbate in the biosynthetic pathway of either of these components. In current studies, changes in hormones and growth factors found in the serum of scorbutic fasting guinea pigs were found to be similar to the changes observed in fasted animals and humans with no ascorbate deficiency. Thus, the growth promoting activity of vitamin C in vivo appears to be mediated initially through reactions other than those directly involved in connective tissue metabolism. These findings represent a major revision in long-standing concepts of the pathogenesis of scurvy. In a second study it was found that the collagen phenotype of a transformed line of Syrian hamster embryo (SHE) fibroblasts was markedly altered from that of normal SHE cells, which synthesized mainly type I procollagen. The observed decrease in total collasen production was primarily due to the complete suppression of synthesis of one of the two subunits of type I procollagen. The major collagenous products synthesized consisted of two polypeptides which are altered proα 2(I) chains that differ from each other.

Continuing progress has been made in the study of the mechanisms of DNA synthesis in mammalian cells. The main approach has been to examine DNA replication in vitro using cDNAs for the replication proteins alpha-polymerase and beta-polymerase, and for a single-stranded nucleic binding protein (helix destabilizing protein). Human and rat cDNAs are being used as probes to study expression of mRNAs to identify polymorphisms in genomic DNA, and to conduct chromosomal localizations. The cDNA to rat brain alpha polymerase has been sequenced. The nucleotide sequence of a cDNA to rat beta polymerase was also determined. and an analogous human cDNA has been isolated and sequenced. The deduced 318 amino acid proteins encoded by the rat and human mRNAs are also similar. It has been possible to isolate and produce mg. quantities of the single stranded nucleic binding protein. Protein-nucleic acid binding studies revealed that the C-terminal domain of the protein is critical for tight binding.

The intracisternal A-particle (IAP) gene is a genetically distinctive type of retrovirus-like element, or retrotransposon, that is extensively reiterated in the genomes of mouse and other rodent species. A cDNA clone encoding an IgE-binding factor (a form of T-cell-secreted IgE-regulatory protein) has been identified as a member of the IAP gene family. In the past year, 5 additional cDNA clones that code for IgE-binding factors were characterized. Each one proved to be a different structural variant of the full-length IAP proviral element. Nucleotide sequencing of a full-size 7.3 kb mouse IAP genomic element has been completed, and compared to the sequence of the Syrian hamster. The data suggests that in the mouse, a cellular form of an IgE regulatory protein gene may have been incorporated into the IAP gag coding region and become part of the 73,000 Da core protein of this defective retrovirus. In other IAP related studies, the mechnism by which methylation inhibits IAP and other retroviral expression is being investigated.

The nuclear antigens that cross-react with an antiserum against the IAP core protein, p73, have been identified. Both these proteins and IAP proteins have binding capacity for single-stranded RNA. Further studies suggest that these nuclear antigens may be produced in rate-limiting amounts in rapidly growing cells. Additional efforts have focused on determining the structure and function of the type II IAP elements.

A large group of interspecific somatic cell hybrids segregating human chromosomes was previously isolated and characterized by isoenzyme and cytogenetic analyses. Further analysis of these hybrid cell DNAs permits the chromosomal, and sometimes subchromosomal localization of human genes. Mapping of several other genes and multigene families is in progress. Several of these probes were also found to identify restriction fragment length polymorpisms (RFLP) which will be useful for genetic linkage studies in families, and for analyzing DNAs from patients with DNA repair defects for the involvement of β-polymerase in these defects. Establishment of genetic linkage maps of human chromosomes 1 and 15 by RFLP analysis of families is also underway. In a second area of investigation, a transforming gene has been isolated from guinea pig leukemia and identified as an altered N-ras gene. Molecular cloning and analysis of the normal and transforming guinea pig N-ras genes is in progress.

The metallothioneins (MTs) are being analyzed by molecular genetic and biochemical approaches as a mode for studying the regulation and function of eukaryotic genes. A mouse nuclear protein that interacts with the heavy metal control sequences of the mouse MT-1 gene has been detected and is being purified. A mouse cell nuclear extract capable of faithful transcription of the mouse M-1 gene has been developed to compare regulatory sequence requirements for efficient in vitro and in vivo transcription, and to assay nuclear regulatory binding proteins. Further studies have shown that the structural basis for differential metal binding by human MT-1 isoproteins is dominated by the carboxy terminal domain of the proteins. Identification of the precise amino acids involved is underway.

In yeast, trans-acting mutants that alter metallothionein gene regulation have been isolated. These mutants are being mapped and the corresponding genes isolated. A substantial number of mutants have been engineered into the yeast metallothionein coding sequence to determine the role of individual amino acids in the structure and function of the protein. Mutants have also been designed to test the copper-binding cysteine residues of the protein. Preliminary studies indicate that these alterations decrease the copper binding properties of the protein in vivo and in vitro, and that different mutations exhibit different quantitative effects.

The objective of another research project is to examine the replication and partition mechanisms that account for the stable inheritance of unit-copy bacterial plasmids. The P1 plasmid prophage is characterized by the presence of iterated DNA sequences at the origin of plasmid replication and in a replication control region, and by a specialized partition apparatus. Information from these studies should provide a better understanding of how DNA synthesis is controlled.

Studies during the past year provided conclusive evidence for the existence of a novel biological control mechanism in which the control region acts in trans without mediation by messenger RNA. Additional studies are aimed at under-

standing the role of multiple origins in a plasmid, by examining the effect of muations that block the major plasmid origin of P1 from functioning. Hybrid strains have been constructed bearing markers that permit selection, counter-selection, and visual discrimination of host mutants altered in plasmid maintenance.

Another approach to understanding the principles of eukaryotic gene regulation has been the analysis of specific protein-DNA interactions in chromatin. A new exoIII footprinting assay has been developed which permits the identification of DNA-binding protein for virtually any gene cloned. The Drosophila heat shock gene activator protein has been purified to homogeneity. This technique has been applied successfully to the Drosophila heat shock TATA box sequence, the rat insulin II gene, and the Drosophila segmentation gene fushi tarazu.

Additional studies focus on mechanisms governing the expression of specific genes in differentiated cells. The level of expression and the appropriate regulation of the various chicken actin genes, when stably introduced into mouse myogeneic cell lines, is dependent upon the developmental history of the particular line. For example, skeletal actin genes are not appropriately regulated in adult myogenic satellite cells, whereas correct regulation is observed in embryonically derived lines. Thus, all the sequence information necessary for the regulated expression of the actin genes is present in the genomic isolates tested. The promoter regions for various actin genes has been identified, and the function of these promoter regions in the regulation of expression of those genes is being analyzed. Additional studies are aimed at defining the essential sequence elements required for promoter function and regulation, and at examining the role of polyadenylation in the regulation of gene expression. The nucleotide sequence of the cytoplasmic myosin gene of Acanthamoeba has been determined and compared with the structure and organization of other myosin genes. Using a probe from the myosin gene of C. elegans, homologous sequences have been identified in the yeast genome.

PC12 cells, derived from the adrenal gland of the rat, undergo neuronal differentiation in response to nerve growth factor (NGF). It has been possible to isolate a cDNA clone and the corresponding genomic sequence that is induced in response to NGF. Antibodies have been made to different portions of the cDNA and are being used to study the induction and localization of the protein. The regulation of expression of the immunoglobulin gene family is being studied by examining the DNA sequence and protein factors that activate expression of immunoglobulin genes. Previous studies showed that specific cells can appropriately regulate the kappa immunoglobulin gene even when not in its normal chromosomal environment. An enhancer element, downstream of the promoter, is necessary for the specific transcription of the gene in myeloma cells. It has now been shown that the promoter is also important in cell specific regulation of transcription of the immunoglobulin gene. The synergism between the kappa enhancer and promoter suggests the existence of a cell specific protein which recognizes both regulatory elements.

Interactions between cytoplasmic myosin and actin are responsible for a variety of cellular motile activities. Current studies focus on the mechanisms by which acto-myosin drives motile activity in non-muscle cells. Using cytoskeletal proteins from various types of muscles, thymus, and brain, it has been possible to show that the regulation of motility in avian smooth muscle and mammalian non-muscle and smooth muscle cells is different. Nonphosphorylated aorta or

thymus myosin (but not unphosphorylated gizzard myosin) induces contraction of myosin-depeleted skeletal muscle myofibrils. Thus, mammalian non-muscle or smooth muscle cells may contain some mechanism other than light chain phosphorylation that regulates actomyosin based motility.

The calcium dependent regulation of enzymes mediated by calmodulin is being studied to elucidate the role of the second messengers, calcium or cAMP, in regulating cell function. During the past year, it was shown that the inositol triphosphate-induced calcium released from the endoplasmic reticulum is modulated by intracellular calcium levels, and that magnesium modulates the interaction of calmodulin with calcium by inducing a large difference in the affinity of calcium binding sites on the calmodulin molecule. This difference in binding affinity helps to explain the stepwise nature of the conformational changes induced by calcium. It was previously shown that different calmodulin-regulated enzymes recognize different domains of the calmodulin molecule and may be activated by different calmodulin conformers. Thus, the stepwise changes exhibited by calmodulin, at different calcium levels, can be used to regulate different metabolic pathways.

The gene of the catalytic subunit of cAMP-dependent protein kinase of Drosophila melanogaster has been cloned and will be used to study the role of cAMP-dependent phosphorylation in this organism. The detailed structure, organization, and possible function of highly repeated DNA sequences are being studied, as well as the mechanisms by which such sequences are amplified either in tandem or through transposition to new genomic loci.

The extraordinary polymorphism of satelllite DNA and the fact that no function can be associated with these sequences suggests that they might be a by-product of DNA replication or recombination in localized chromosomal regions. An apparently single-copy DNA sequence that neighbors satellite DNA was cloned from the African green monkey genome. Analysis of genomic libraries from human and mouse indicate that both the single-copy sequence and its association with species-specific satellite DNA are conserved in mammalian DNA. However, two types of cloned genomic segments were obtained from the human and monkey, only one of which is colinear with the genomic arrangement of the sequences. The most likely explanation is that the cloned segments undergo rearrangements during cloning in E. coli when both the satellite and the single-copy sequence has been localized on human chromosome 4. In other studies, the remarkable evolutionary fluidity of satellite DNA is being used to analyze the evolution- and taxonomic relations among the carnivores in the felid and canid families.

SINES are short repeated segments dispersed throughout the genome. A SINE family unique to carnivores has been isolated and characterized from Felis catus. This sequence is organized differently in different canid species and can be used to distinguish between them. Cloned ALU sequences (the typically primate SINE family) have been used to detect and purify sequence-specific binding protein in nuclear extracts from primate cells. Exonuclease III protection studies indicate that the binding site is located downstream of the A-box sequence associated with the RNA polymerase III promoter found on ALU sequences. To understand the significance of the LINE-1 family of interspersed repeats, the potential for some family members to be functional genes and encode a protein is being investigated. cDNA clones representing polyadenylated, cytoplasmic LINE-1 RNA from human teratocarcinoma cells have been isolated. The cDNA sequences have defined a subset of LINE-1 sequences that appear to be specifically transcribed (or processed) in these cells.

Improved methods for the specific isolation of antigen reactive cells are being developed in order to study the molecular interactions that occur in their immune reactions. Recent investigations have focused on a possible requirement for class II major histocompatibility complex (Ia) molecules (either on syngeneic accessory cells or on allogeneic stimulators) in the initial activation of cytotoxic T lymphocyte precursors (CTLp) for allocytotoxic responses. Earlier experiments had indicated that such cells were best triggered into mature CTL in vitro by cells known to be Ia+. However, further experiments have now shown that the early activation of CTLp is independent of Ia+ syngeneic accessory cells.

"Xenogenization" (induced mutation in tumor cells to increase their immunogenicity) is a potential approach to cancer immunotherapy of tumors that fail to elicit an effective immune response. The humoral response in mice to a chemically xenogenized murine lymphoma cell line has been further characterized. Antibodies of both IgG and IgM classes capable of binding the xenogenized tumor could be detected after a single sensitization and reached a maximum after 3-4 injections. The novel determinants recognized on the xenogenized cells were not close to those shared with the parental tumor. Thus, chemical xenogenization of a murine lymphoma leads to development of novel determinants detectable by specific antibodies.

Another approach to the immunotherapy of tumors is the immunochemical attachment of antigens to tumor cells in order to increase their susceptibility to cellular immune reaction. Antibodies to public specificities of class I major histocompatibility complex molecules are used to attach allogeneic affinity-purified molecules to mouse lymphoid tumor cells. Rabbit polyclonal anti-mouse class I MHC antibody can successfully bind allogeneic class I molecules to the cell surface. Procedures for the effective use of monoclonal antibodies are being sought, and the use of class I antigen-coated tumor cells as immunogens and targets for cellular immunity is being studied.

In another project, the biochemical basis for defective differentiation in granulocyte leukemia is being sought. In previous studies it was shown that the maturation of neutrophil precursors from guines pig bone marrow is dependent on a "neutrophil precursor maturation factor" (NPMF), that is associated with transferrin. In order to further test the homogeneity of this factor, and to assess its binding to normal and leukemic neutrophil precursors, monoclonal antibodies are being made. Studies have been initiated to develop an in vitro maturation assay for human neutrophil precursors corresponding to that developed for guinea pig cells. As in the guinea pig system, the matur- of human neutrophil precursors in the presence of human serum is accompanied by a pronounced increase in alkaline phosphatase activity. Screening of serum chromatographic fractions for NPMF activity is now underway using alkaline phosphate activity as a marker of maturation induction in these cultures.

Using the combined high performance liquid chromatography (HPLC)-SDS gel electrohporesis procedure previously reported, five patients with chronic myelocytic leukemia (CML) have been followed for several years from the stable phase to the blast crisis stage of the disease. The results indicate that prior to the onset of blast crisis there is a progressive decrease in both cell membrane and granule phenotypic proteins in mature granulocytes. An attempt is being made to identify the genes in CML myeblasts that are responsible for leukemogenic activity.

Ion-exchange displacement (IED) chromatography is being developed for the fraction of macromolecules and particles of biological interest, employing carboxymethyldextrans (CM-Ds) displacers. The procedure is particularly advantageous when large amounts of source material must be used to obtain sufficient amounts of a minor component, since the resolving power of the system can be focused on the narrow range of affinity represented by the protein of interest and its nearest neigbors. However, it is also applicable to ion-exchange HPLC. Recent efforts have been directed toward the simplification of the preparation of the displacers.

The Laboratory of Cellular Oncology (Dr. Douglas Lowy, Chief), conducts fundamental research on the cellular and molecular basis of neoplasia. The p21 *ras* oncogenes and papillomaviruses have provided a model system for studying the mechanisms by which tumor viruses or cellular genes contribute to oncogenesis. The goal of these experiments is to devise approaches to prevent or reverse such changes in cells. An inframe mutational analysis of the v-*ras*H oncogene of Harvey murine sarcoma virus (Ha-MuSV) has indicated that multiple regions are required for transforming activity and nucleotide binding. A new class of mutants has been identified whose lesions appear to define a region of *ras* that may interact with its cellular target. In vivo studies of Ha-MuSV have shown that deletion of a segment of non-*ras* viral sequences changes the type of tumor induced by the virus. This change in target cell specificity appears to be associated with the presence of an enhancer element located in this region.

In studies of the bovine papillomavirus (BPV) genome, the second transforming gene of this virus has been identified as being encoded by the E5 open reading frame. The E1 gene normally inhibits the efficiency of cell transformation by the viral genome. A peptide encoded by the E2 open reading frame binds directly to several sites within the upstream regulatory region of the BPV genome suggesting that the enhancer activity of the E_2 gene product is mediated via the direct interaction with the viral regulatory region.

Continuing studies on oncogenesis are providing more information on the mechanisms of gene regulation associated with cell differentiation and neoplasia. The possible activation of cellular oncogenes in the spontaneous Hodgkins-like reticulum cell neoplasm of the SJL/J mouse has been analyzed. The data from recent studies suggests that the development of this neoplasm may be associated with the activation of c-*abl*. The role of *mos* in the anchorage independent (AI) growth and metastatic potential of S+L-mink cells superinfected with a novel dual tropic mouse retrovirus was studied. The results indicated that increased amplification of v-*mos*, and enhanced expression of its mRNA may be important in the AI phenotype, oncogenicity, and metastatic ability of S+L-mink cells in this system.

Another project is designed to investigate the molecular genetic mechanisms of neoplastic transformation of normal tissues and the cellular immune defense mechanisms against neoplastic cells. A human hepatocarcinoma oncogene (hHC) has been isolated which transforms NIH3T3 cells at a very low efficiency. Its transforming capability can be enhanced with AFB-1-epoxide activation. Nucleotide sequence analysis has indicated the presence of an open reading frame that could encode a peptide of 155 amino acids. Additional studies should reveal whether this gene is related to previously described oncogenes.

The functional relationship between two lymphokines, IL-2, and cytotoxic cell differentiation factor (CCDF), has been studied. In the absence of antigenic or mitogenic stimulation, IL-2 can provide the first signal to activate the differentiation of cytotoxic effector cells only in the presence of CCDF. The synergistic action of CCDF and IL-2 is critical for the generation of lymphokine-induced cytotoxicity, and thus may play a pivotal role in host defense mechanisms against tumor cells. The virus-negative methylcholanthrene induced BALB/c sarcoma, Meth A, can be passed both in vivo and in vitro, allowing for comparison of the response in cell culture with that in the intact mouse. This test system makes it possible to analyze the direct action of interferon (IFN) on Meth A cells separately from the host defenses. Cells treated in vitro were analyzed for the anti-cellular effects in vitro and immune reactivity in vivo. At the concentrations tested, IFN was cytostatic, but not cytotoxic. When normal Balb/C mice were challenged with IFN treated Meth A cells, the development of tumors could be prevented as long as the challenge dose was 10^5 cells or less. However, the protective effect of IFN was negated in immunodeficient mice. These experiments confirm the necessity of functional T cells in order for IFN to exert its antitumor effect, suggesting that the major effect of IFN on chemically induced sarcomas is mediated through the host immune response, rather than by its anti-cellular activities.

Continuing progress has been made in elucidating the biological function(s) of the normal human c-fps/fes proto-oncogene, and in understanding the molecular and cellular basis of its oncogenic potential. A cloned human genomic DNA fragment containing c-fps/fes-homolgous sequences contains the entire coding sequence of the human c-fps/fes-encoded protein. This protein, a 92 kda cellular tryosine kinase, is the gene product of human c-fps/fes. A new cellular tryosine kinase present in all vertebrate cells has been identified that is immunologically related to the c-fps/fes product, but is encoded by a different gene. Characterization of this protein has been initiated.

The Laboratory of Genetics (Dr. Michael Potter, Chief), conducts studies of how specific genes and their products determine and regulate biological functions involved in neoplastic development. A major segment of the work is devoted to a continued understanding of immunoglobulin genes. The induction of plasma cell tumors in mineral oil treated BALB/c mice provides a model system for studying the basic pathogenetic mechanisms that are involved in many forms of leukemias and lymphomas in man, as virtually all of these tumors are associated with a non-random chromosomal translocation. In addition, susceptibility to plasmacytoma induction in mice is genetically determined and is not a general characteristic of the species. Recent studies have shown that plasmacytoma susceptible BALB/cAn mice slowly repair double-stranded chromosomal breaks when compared with plasmacytoma resistant DBA/2 or CDF_1 hybrids. This may be the first evidence of DNA-repair defect in mice. Further studies should yield more information on the genetic basis for the difference. Another approach to testing the role of DNA damage and repair in plasmacytoma susceptible and resistant mice, has focused on the role of nuclear poly (ADP-ribosylation) (pADPR) in the DNA repair process. pADPR has been purified to near homogeneity. Monoclonal and heterologous antibodies are being prepared to this enzyme.

A recent study on the histogenesis of plasmacytoma development in BALB/c mice has been conducted. It has been shown that proliferating plasma cells arise throughout the latent period, before the development of plasma cell tumors. Comparable lesions do not (or rarely) develop in resistent strains. Thus,

susceptibility is correlated with pre-neoplastic changes suggesting that susceptibility and resistance genes are expressed very early in plasmacytoma development.

A new method for accelerating plasmacytoma induction in BABL/cAn mice has been developed which involves infecting pristance conditioned mice with transforming retroviruses containing specific types of modified myc oncogenes. Thus far, the retroviral construct J-3 which carries a hybrid avian V-myc gene, has been identified that induces the expected yield of plasmacytomas with short latent periods. In contrast, other viral constructs carrying myc sequences induce predominantly myeloid tumors. Experiments are in progress to determine the basis for the lymphocytic tropism. Most pristane/J-3 induced plasmacytomas lack chromosome 15 translocations but produce v-myc transcripts. This method should allow us to reconstruct critical biochemical changes in plasmacytomagenesis by supplying appropriate genes.

A factor required for plasmacytoma growth in vitro has been purified to apparent homogeneity. Experiments are currently in progress to obtain animo terminal sequence data which will be used to generate a synthetic oligonucleotide probe for gene cloning. Attempts are also underway to raise monoclonal antibodies to this factor.

Significant progress has been made in characterizing the oncogene deregulation that occurs in neoplastic cells. The major emphasis has been on c-myc, c-myb, and ras genes. A series of myeloid tumors (formerly called ABPL) which have Moloney leukemia virus inserts in the 5' end of the c-myb locus have been characterized. It has been shown that these are myelomonocytic cells. When these cells are stimulated by factors that normally induce terminal differentiation, the abnormal myb genes are activated, which appears to keep the cells in a proliferative mode. Further studies on the molecular basis for regulation of expression have focused on two unusual plasmacytomas that produce excessive amounts of very large c-myc transcripts. The c-myc genes in each of these tumors carries a mutation that exerts its effects during post-transcriptional stages of c-myc expression by prolonging the half-life of the myc RNA. A series of genes whose products participate in defined, but different, immunological functions are being characterized. Genes encoding members of a kappa chain subgroup have been cloned from recombinant DNA libraries generated in six different wild mouse species. Sequence analysis of these genes is currently in progress. Results from these studies should provide additional information on the mutational mechanisms operating on these genes in natural populations during a relatively short evolutionary period.

Model antibodies directed against the model protein antigen, isozyme c, are being used as probes to study antibody-protein interactions and structure-function relationships, and to study developmentally regulated antigens in normal and neoplastic development. The interaction of antibodies with the epitopes are modelled and ultimately refined by protein crystallography studies.

The primary structures of three monoclonal antibodies to hen egg white lysozymes (HEL) have been determined and the respective epitopes on HEL have been identified. Crystallization of the Fab or Fab-HEL complexes has produced suitable crystal structures of HyHEL-5-HEL that can be used to directly define the interaction. The computer fitted complex of this antibody with the proposed epitope in lysozyme indicates a very extensive contact interface involving

antibody framework residues as well as 5 out of 6 complementarity determining regions. This indicates the extraordinary specificity and strength of monoclonal antibody-protein antigen interaction.

Investigations on the acute erythroleukemia-inducing virus, spleen focus-forming virus (SFFV), have concentrated on determining which viral sequences are responsible for pathogenicity and erythroid cell specificity. The data have shown that sequences in the long terminal repeat (LTR) region of SFFV do not determine the erythroid cell tropism of the virus and that expression of the SFFV env gene, in the absence of other SFFV genes and the SFFV LTR, is pathogenic. Thus, the primary effect of SFFV on erythroid cells is due to the product of its env gene. To further understand how the viral env gene product may do this, variants of the virus, $SFFV_P$ and $SFFV_A$, which induce actue erythroleukemia in mice have been studied. These viral variants differ in their effects on erythroid cells as well as in the post-translational modification of their env gene products. By making recombinants between $SFFV_P$ and $SFFV_A$, it has been possible to localize the sequences responsible for the biological and biochemical differences between these two viruses to a specific region in the envelope gene.

Studies on the genetics of susceptibility to early erythroleukemia induced by Friend murine leukemia virus are concentrating on a gene (located on chromosome 5) that encodes a viral envelope protein. Attempts are being made to confer resistance to leukemia by genetic transfer of this gene, as well as to apply this mouse model for resistance to the treatment of human diseases such as AIDS.

A separate research project has concentrated on developing new and difficult systems for cell culture. The major emphasis is on the culture of cells from the neonatal rat olfactory epithelium (OLFE). Development of this system will make available the first mammalian neuroblast to neuron cell culture.

The activities of the Laboratory of Mathematical Biology (Dr. Jacob Maizel, Chief), encompass studies of macromolecular structure and function, membrane structure and function, immunology, pharmacokinetics, and computational and modeling methodology. Application of theoretical understanding to these biological systems is accomplished through the use of advanced computing. Computerized analyses is being applied to the study of various virus-cell systems. Sequences of picornaviruses are examined for relationships within the family and to other known and hypothetical proteins. Studies of adenoviruses are providing information on early events in virus replication in which the cell's metabolism is subverted to viral functions, and late events, during which assembly and morphogenesis occurs.

New analyses of proteins and nucleic acids are developed and implemented. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. New sequences are compared with computerized databases to detect relationships with known proteins. A government-owned, contractor-operated supercomputer laboratory has been established at the Frederick Cancer Research Facility under the sponsorship of this Laboratory. Equipment consisting of a Cray XMP supercomputer, VAX 8600 and 785 front end and graphic workstations became fully operational on April 1, 1986. medical problems. This machine is the first scientific computer dedicated entirely to biomedical research.

The computational scheme used to associate α-helical amphipathicity with known immunodominant helper T-cell antigenic sites in proteins, has been modified to produce algorithms for predicting T-cell antigenic sites. The algorithm identifies segments of a protein that would have a high probability of being an immunodominant T-cell site in the protein. The main characteristic of such a protein segment is that, if isolated as a polypeptide, it could form an amphipathic α-helix. Other correlates have been identified and will be incorporated into future algorithms. The algorithms have been developed and tested, and are being used to predict likely sites for T cell recognition of AIDS virus antigens and malaria parasite antigens. Some of the first peptides predicted this way are being made and tested.

Studies of biological macromolecules and their properties have progressed. Both long range and short range interactions in proteins have been investigated by tabulating data from X-ray crystal structures. Hydrophobicity scales and effective interaction energies between all types of pairs of amino acids have been derived and are being applied to locate hydrophobic nuclei and to attempt to incorporate them into a protein folding scheme. Molecular modeling has focused on membrane channels, DNA local conformations, DNA-carcinogen interactions, and DNA-protein interactions. Proteins that have been modelled are acetylcholine receptor, interleukin-2, δ hemolysin, α toxin and sodium action potential channel. For DNA-protein interactions, the strengths of various types of DNA-protein are evaluated. The repressor is placed in the most favorable orientation within the major groove of the operator region. Subsequent comparisons are made with experimental binding constrants of various operators and their mutants. This combination of modelling with experimental data has permitted a first derivation of detailed interatomic interaction energies from experimental binding constrants.

Development and implementation of a general purpose computer system (SAMM/CONSAM) for the simulation, analysis, and modelling of physiological systems has continued. Examples of SAAM/CONSAM development and application include studies of in vivo transport of free cholesterol and esterfied cholesterol among the components of blood. The metabolism of apolipoprotein B (apoB) in humans has been quantified more precisely using an integrated model which was developed for the analysis of the isotope kinetics of apoB in very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). A detailed quantitative description of the metabolism of the core lipids of chylomicrons has been proposed and is being tested in a rat model. LDL ApoB turnover kinetics has been re-examined. A new model of LDL metabolism was developed and is being tested. In this study, the model underscores the physiologic importance of cholesterol-ester exchanges in the production and metabolism of H-LDL and L-HDL. Selenium kinetics may be important in cancer treatment and prevention. A selenium model is being tested using the pilot study data for oral selenate dosing.

A continuing research interest deals with the insertion and organization of proteins and lipid molecules in membranes; interactions of membranes with toxins, viruses, channel proteins and polypeptides, and membrane fusion. Spectroscopic techniques are used to study lipid-protein interactions and membrane fusion, and conductance across planar black lipid membranes (BLMs) is measured to approach questions of membrane destabilization and channel formation.

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

Studies on channel-formation with cytolysin isolated from large granular lymphocyte tumors and from cytotoxic T lymphocytes are being pursued with lipid vesicles and planar bilayers to examine the mechanism of its lytic action. Recently it has been predicted that Interleukin-2 (IL-2) might form channels in bilayers, and that this might have important implications for the biological mechanism of this molecule. The interaction of adenovirus with lipid membranes has been studied in order to elucidate the mechanism by which adenovirus enters the cytoplasm of the host cell. The results indicate a pH-dependent permeability change in the liposomes, which parallels the pH-dependence of the disruption of endocytic vesicles by adenovirus.

Another ongoing study has demonstrated the feasibility of delivering monoclonal antibodies via lymphatic vessels for diagnosis and possible treatment of lymph node metastases and lymphoma. The initial animal studies proved promising, and clinical studies are underway to test the efficacy of lymphatic delivery in melanoma, T-cell lymphoma, Hodgkin's disease, small cell carcinoma, non-small cell carcinoma, and breast cancer. 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.

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. These theoretical predictions are being tested experimentally. An algorithm has been developed with which to analyze secondary structural characteristics of peptides and to assess their potential for antigenicity. The results are being applied to HTLVIII/LAV envelope protein.

Analysis of large 2D gel data bases has continued, as well as the development and implementation of new algorithms for the detection of such subtle changes as charge pairs, etc. Expansion of GELLAB by use of SAIL constructs with subsequent translation continues to improve this system.

Work on nucleic acid structures has involved studies of global sequence homology, the study of nucleic acid molecular helical twist, torsion angle, etc., and the analysis of structure related to predicted shapes and gel migration patterns. Work is now beginning on the development of a Nucleic Acid Expert System, which would be directed to both intelligent queries by researchers, and a non-ad hoc structuring of the relationships among the various software/hardware complexes available at the Frederick center.

Research interests in membrane biology address problems related to the topology, structure, and dynamics of plasma and intracellular membranes as well as of intracellular junctions. The compactness and cytochemistry of cytoplasmic and extracellular matrices are investigated using techniques of freeze-fracture cytochemistry.

The Laboratory of Pathology (Dr. Lance Liotta, Chief), provides diagnostic services in anatomic pathology, cytopathology, electron microscopy, and hematopathology for the Clinical Center of the NIH, and has research programs in various areas of experimental pathology. Approximately 6,000 surgical specimens of biopsies (approximately 60,000 slides) were accessioned during the past year. Approximately 1,000 specimens of fresh human tissues were furnished to NIH scientists in various laboratories. Clinicopathological studies on various neoplasms, acquired immune deficiency syndromes, and other diseases are in progress. The Cytopathology Section, which provides diagnostic services in cytology and medical genetics, accessioned approximately 6,000 cytology specimens during the past year. Recent interests have included the application of immunoperoxidase techniques to the cytologic diagnosis of malignant lymphoma.

The Ultrastructural Pathology Section, which provides diagnostic electron microscopy services for the Clinical Center, accessioned approximately 300 cases, over 200 of which were processed and diagnosed. Research efforts in this section have led to the development of new methods of classifying neuroblastomas. It was shown that peripheral neuroepithelioma, a unique, previously uncharacterized tumor of childhood, is distinct from classic childhood neuroblastoma, in that it lacks N-myc expression, and instead, routinely demonstrates a reciprocal (11:22) chromosomal translocation. C-myc is expressed routinely by this tumor and, unlike most neuroblastomas, it does not produce catecholamines but instead contains purely cholinergic neurotransmitter enzymes. Clinical investigations based on these observations are underway, and early results indicate a prognosis inferior to Ewing's sarcoma (with which it is often confused) and vastly superior to disseminated neuroblastoma (which was the historical diagnosis).

Similarly, the development of improved methods of immunocytochemistry, tissue culture, and monoclonal antibody techniques has refined the classification of childhood rhabdomyosarcoma. Two basic types of rhabdomyosarcoma have been identified, and in a retrospective analysis of 159 cases, these new methods of classification were shown to be of prognostic value. Previous studies have shown that the type of matrix molecules produced by pediatric "round cell" tumors are indicative of their cell of origin. A previously undescribed high molecular weight matrix protein has been identified and characterized, and further studies of its function and composition are underway. A neurone-specific enolase was shown to be a reliable marker for primative childhood tumors of neural origin, and thus, is useful in the differential diagnosis of neuroblastoma versus other solid tumors of childhood.

Research on the immunochemistry of complex carbohydrates has focused on the determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures by gas chromatography/mass spectrometry (GC/MS), the development of hybridoma antibodies against oligosaccharide haptens, and immunochemical studies of cell surface glycoproteins. It is anticipated that the analysis of the repertoire of oligosaccharide chains produced by cells or tissues will reflect states of cellular differentiation and reveal potential cell surface markers.

Membrane glycoproteins behave as either carbohydrate or peptide antigens and occasionally express antigens that combine specific structural elements from both sugar and peptide moieties. Studies are underway to characterize the fine specificities of autoantibodies against glycoproteins of human erythrocytes from patients with altered immunologic states. In addition, monoclonal antibodies

are being prepared against various portions of the carbohydrate and peptide moieties of the major sialoglycoprotein of human erythrocytes.

The mouse immune response to streptococcal group A carbohydrate (GAC) utilizes many different gene segments from a diverse array of precursor Vh genes. The gene family number is estimated at 10 and the diversity of response in normal mice at over 200 different antibodies. Hybridoma derived cell lines making antibodies bearing Vh determinants of the GAC-J606 family derived from Balb/c and CBA/N mice are being used to obtain deduced sequences of heavy and light chains of their antibodies. This model provides an ideal system in which to investigate possible constraints on gene segment utilization, and to enhance our understanding of the organization and expression of the immune repetoire of antibodies.

A major research effort within the laboratory has been to identify and characterize the biochemical and molecular genetic mechanisms involved in tumor cell invasion and metastases formation. Three biochemical factors have been identified that are quantitatively enhanced in actively invading tumors cells: a) specific new types of proteases, b) the laminin receptor, and c) a new type of autocrine motility factor (AMF). Antibodies against these proteases react with actively invading breast carcinoma cells in tissue sections, and thus, are of diagnostic usefulness. Further, blocking the proteases inhibits tumor cell invasion in vitro.

A major objective in these studies has been to analyze the events that take place at the interface between the tumor cell and the basement membrane, and to elucidate their role in the invasive process. Laminin, a glycoprotein of the basement membrane plays a major role in metastatic tumor cell attachment. The specific domain of the laminin molecule that attaches to the tumor cell has been identified, and examined by rotary shadowing electron microscopy. The cell membrane receptor for laminin has been identified and characterized. A fragment of the laminin molecule which binds to the receptor and blocks attachment will inhibit metastases formation in animal models. Laminin receptors can be readily measured in surgical specimens of human breast cancer tissue, and recent studies have shown that a high content of exposed laminin receptors generally correlates with increased lymph node metastases in these patients. Clinical trials are being initiated to study the localization of metastases using labeled laminin fragments. Monoclonal and polyclonal antibodies have been prepared against the human breast cancer laminin receptor. These antibodies have provided new information about the distribution and function of the receptor. In addition, they have been used to screen a cDNA clone from the human gene coding for the receptor. Further characterization of this genomic clone is underway.

Type IV collagenase is an important basement membrane degrading metalloproteinase that malignant cells produce and secrete to facilitate traversal through blood vessel walls. Type IV collagenase is markedly augmented following ras oncogene transfection. Human type IV collagenase was isolated from culture supernatants of metastatic melanoma cells and is being characterized. A new collagen IV binding protein has been identified that has no collagenolytic activity and does not possess any characteristics of known matrix proteins. Malignant tumor cells must be highly motile while invading tissue and metastasizing to distant sites. A number of metastatic cell lines produce and respond to autocrine motility factors. AMF has been purified from the conditioned media of a human melanoma cell line and is being characterized. Transformed 3T3 cells

produce and respond well to autocrine factors but poorly to platelet-derived growth factor (PDGF), while non-transformed cells (which do not produce AMF) respond well to PDGF but less well to the AMF produced by the transformed cells. This suggests that the abiility to produce and respond to autocrine motility factors is an important characteristic of the metastatic phenotype.

A variety of techniques are being used to identify specific genetic elements whose expression is altered in metastatic cells. Several cDNA probes were tested for their relative expression in RNAs from a series of related murine melanoma cell lines that differ in metastatic potential. One cDNA probe, pNM23, was found that can identify mRNAs differentially expressed by cells of varying metastatic potential both in vitro and in vivo. It appears that metastases may not result simply from the acquisition of invasive and other traits, but may also involve the loss of certain cell functions.

The ability of $\underline{ras}^H$ genes to induce metastatic potential as well as transformation and tumorigenicity has been demonstrated in several cell types. NIH-3T3 cells transformed with the cloned T24 human $\underline{ras}^H$ oncogene resulted in cells which formed lung metastases. The $\underline{ras}^H$ oncogenes can also induce metastatic behavior in diploid fibroblasts. However, transformation itself or the ability to grow as a tumor, does not always result in metastasis. The normal cellular counterpart of the $\underline{ras}^H$ oncogene can also transform NIH-3T3 cells under certain conditions, however, these transformed cells, while highly tumorigenic, do not metastasize. Similarly, $\underline{ras}^H$ did not induce metastatic potential in a murine epithelioid line. These cells, although morphologically transformed and highly tumorigenic, did not metastasize. The induction of metatatic potential in those cells is being attempted in gene transfer experiments.

The Laboratory of Pathology conducts an active research program on the immunological classification 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, to distinguish new clinicopathologic entities, and as a basis for potential immunotherapy. For example, a classification of post-thymic T-cell malignancies has been proposed which delineates at least five major clinicopathologic entities with differing clinical presentations and prognosis.

Another study demonstrated that in some patients with follicular lymphoma, (a monoclonal B-cell tumor), mature T cells were numerically predominant, suggesting that these infiltrating non-neoplastic T cells may in fact represent a positive host immune response. These studies suggest that the neoplastic cells of follicular lymphoma are susceptible to host immunoregulation.

A new research program has focused on studies of the molecular basis of the diagnosis of human lymphoproliferative disease. The T- and B-cell lineage of diffuse, aggressive lymphomas can be confirmed by detection of appropriately rearranged T-cell receptor or immunoglobulin genes. Moreover, molecular genetic analysis has been used to identify malignant lymphoma in the tissues of patients which lacked histologic evidence of malignancy. A subclass of diffuse, aggressive lymphomas that contain the t14, 18 chromosomal translocation characteristic of follicular lymphoma has been identified, suggesting that many of these high grade B-cell lymphomas may originate as follicular lymphomas both clinically and at the molecular level.

Monoclonality and T-cell lineage were also demonstrated by rearrangement of the T-cell receptor gene in Tγ lymphoproliferative disorder, (in which a neoplastic basis had been questioned), Angio-immunoblastic lymphadenopathy, which frequently progresses to malignant lymphoma, was shown to contain monoclonal rearrangements of either immunoglobulin genes, T-cell receptor genes, or both simultaneously.

In follicular B-cell lymphomas, unlike most malignant lymphomas, there is a high frequency of clonal evolution. This allows for the escape of the malignant cells from identity by anti-idiotypic antibody and from detection by the host immune response. Likely mechanisms of clonal evolution include somatic mutation of a V region gene, new variable gene rearrangements, or heavy chain constant region isotype switching. Currently it is thought that there is conservation of the immunoglobulin allele involved in the t14, 18 translocation characteristic of follicular lymphomas, although new arrangements may continue to occur in the productive allele. These observations may impact treatment strategies for these lymphomas which, although clinically low-grade, have been refractory to cure with current regimens.

An active research interest continues in the regulation of cell growth by transferrin receptors (TFR). Normal cells regulate transferrin receptor appearance by carefully controlled tissue-specific growth factors and their receptors. However, in malignant cells this regulation is lost and transferrin receptor expression becomes constitutive. Elucidating the molecular mechanisms regulating the TFR genes will provide a better understanding of normal growth regulation as well as what may lead to uncontrolled proliferation.

A new technique has been developed for the rapid identification of sequence-specific DNA binding proteins to examine mammalian transcriptional regulatory factors. The utility of this method has been demonstrated using the lambda repressor, another well-characterized DNA-binding protein, as a model. This approach has led to the identification of a cellular factor which binds to the enhancer of the Gibbon ape leukemia virus. In another application of this method it was shown that there is a region upstream of the c-myc oncogene which negatively regulates transcription from promoter P1 and P2.

The Dermatology Branch (Dr. Stephen Katz, Chief), 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 provides dermatology consultant services to the NIH Clinical Center (approximately 1500 patients are seen each year).

A major area of study is the role of the epidermis as an immunological tissue. When epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells. In recent studies, it was shown that when these cultured Langerhans cells are modified with hapten, they can generate primary immune responses, and that sensitized T cells thus generated respond preferentially to the same hapten in vitro in secondary cultures. Murine dendritic thy 1 positive epidermal cells have been extensively studied and characterized. In a series of studies in mice it was shown that these cells are of T cell lineage, even though they do not express mature T cell markers. When thy 1^+ dendritic cells from the skin of nude mice are cultured, they express the entire T cell antigen receptor in their membrane. Thus, these

cells may represent an extra-thymic source of T cells in nude as well as normal mice. Epidermal Langerhans cells (in contrast to keratinocytes) express little or no major histocompatibility Class I antigen. This may have relevance to both the immune response in the skin as well as to mechanisms of skin allograft rejection.

Another research project examines the molecular basis of autoimmune skin diseases. The goal of this work is to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis and epidermal basement membrane zone (BMZ). The antigens defined by three of these diseases have been identified and characterized. The binding of antibody to these antigens has been utilized to make a diagnosis in various complicated cases of these diseases. Studies are underway to try to characterize some of the antigens defined by these autoimmune diseases at the gene level.

Complement derived anaphylatoxins play an important role in the inflammatory response and in immunologically mediated skin diseases. The human anaphylatoxins C5a and C3 have been isolated, and extensive clinical testing of this material in the skin of humans is being carried out. The ability of anaphylatoxins to modulate cell surface receptors for immunoglobulin and complement on the surface of leukocytes is also being studied.

Increased evidence indicates that under certain conditions, human endothelial cells can become immunologically competent. Dermal microvascular endothelial cells have been isolated from human skin. These cells are the targets of cutaneous or systemic necrotizing vasculitis. They proliferate wildly in vascular tumors such as Kaposi's sarcoma and may be the earliest targets in skin graft rejection. It should now be possible to analyze the immunological phenotype and immunological potential of these cells. Recently it was shown that endothelial cells derived either from umbilical veins or from the dermal microvasculature can be induced to differentiate in vitro on selective matrices and under certain culture conditions to form tube-like structures resembling blood vessels.

Clinical evaluation of the safety and effectiveness of new oral and topical agents, particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne continues. 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. When treatment was stopped, the patient with the nevoid basal cell carcinoma syndrome developed more than 20 tumors within one year, while there has been no new tumor formation in the patient with arsenic-induced tumors as of the 6-month visit. The efficacy of isotretinoin as a chemopreventive agent is being studied further in a series of patients with xeroderma pigmentosum and nevoid basal cell carcinoma syndrome. Preliminary results indicate only partial efficacy in inhibiting new tumor formation. Severe spinal ligament calcification was observed in some patients with multiple basal cell carcinomas treated with high doses of isotretinoin for a minimum of two years. In contrast, extraspinal tendon and ligament calcification was observed after chronic therapy for psoriasis and disorders of keratinization with the aromatic retinoid, etretinate.

Studies continue on the role of DNA repair processes in cancer, aging and neurodegenerative disorders. DNA damage caused by ionizing-radiation-induced

free radicals or free radicals induced from normal intracellular metabolic reactions may be a causative factor in cancer, aging, and the neurodegenerations of Alzheimer's disease and Parkinson's disease. In the past, it has been very difficult to identify the types of free-radical induced DNA base damage that occurs in intracellular DNA. A new technique using gas chromatography-mass spectrophotometry (GS-MS) has been developed which does not require the use of radioactivity and should, therefore, make it possible to identify the many types of possible damage to each of the four DNA bases. Identification of such lesions would permit studies to determine their biological effects and whether they are subject to various DNA-repair processes in normal and patient (e.g., Alzheimer's disease) cells. Using the GC-MS techniques, a novel purine lesion induced by ionizing radiation in the DNA of normal human lymphoblastoid lines has been identified. The next step will be to see if normal cells can repair this particular type of DNA damage.

Work continues on the physico-chemical structure of keratin and other type filaments. The availability of complete amino acid sequences of several keratin chains has led to the development of more advanced structural models of intermediate filament structure. 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 tachtoid structures in which their rod domains are loosely arranged in an open mesh-like network, similar to their observed structure in vivo. Structural studies have permitted the construction of models on how this network may be assembled, and thus regulate gene expression.

The Metabolism Branch (Dr. Thomas Waldmann, Chief), conducts clinical and basic laboratory research studies designed to define host factors that result in a high incidence of neoplasia. A broad range of immunological investigations are carried out in patients with immunodeficiency diseases and a high incidence of neoplasia, as well as in patients with malignancy, expecially T- and B-cell leukemias. A second area of interest focuses on determining the physiological and biochemical effects that a tumor produces on the metabolism of the host.

Previously, recombinant DNA technology was used to analyze immunoglobulin (Ig) gene rearrangements to classify neoplasms previously of uncertain lineage, to aid in the diagnosis of neoplasms of the B-cell series and to define the state of differentiation of neoplastic B-cell precursors. During the past year, progress has been made in the molecular genetic analysis of the genes encoding the T-cell antigen receptor. The goal of these studies is to provide an analogous marker for T-cell clonality and lineage. The T-cell lymphocyte antigen specific receptor is a 90 kd heterodimer consisting of 40-50 kd $T\alpha$ and $T\beta$ subunits. Like immunoglobulins genes, the genes encoding the subunits undergo somatic rearrangements in the course of lymphocyte ontogeny. A third T-cell gene ($t\gamma$) has been identified which has many properties in common with $T\alpha$ and $T\beta$ genes. The T-cell alpha, beta and gamma receptor genes are located on chromosomes 14q11, 7q32-35 and 7p13-15 respectively. The T-cell lines and leukemias derived from patients with ataxia telangiectasia have a high incidence of chromosomal translocations and breaks at these sites, and these patients appear to have a defect in DNA repair.

Probes to the genes encoding the alpha, beta and gamma chains of the T-cell antigen receptor and to immunoglobulin genes have been utilized to assess

clonality, lineage, and state of maturation in lymphoid malignancies. A series of leukemias of B and T cells at various stages of maturation were analyzed for their receptor gene arrangement and expression into mRNA. These studies suggest that the majority of mature leukemias have rearranged immunoglobulin or T cell receptor genes, but not both. In contrast, 5 of 9 B cell precursor leukemias and 1 of 4 pre T cell leukemias rearrange both J_H and T cell receptor genes.

Using a combination of receptor gene rearrangements and mRNA expression, the spectrum of leukemias studied within the T cell series permitted an assignment of the order of T cell gene rearrangements with $T\gamma$ gene rearrangement proceeding $T\beta$, and $T\beta$ rearrangements proceeding $T\alpha$ expression. In these studies, Southern blot analysis was sufficiently sensitive to detect even minority (2-5%) populations of clonal cells. Thus, analysis of T cell clonality can aid in the diagnosis of T cell malignancy, and in following the efficacy of therapy of T cell leukemias and lymphomas.

Many mature B-cell malignancies manifest chromosomal translocations that involve the immunoglobulin gene locus. These translocations play a role in malignant transformation, and may be of value in the diagnosis and monitoring of the therapy of these malignancies. The t(14;18) (q32;q21) chromosomal translocation characteristic of over 80% of follicular B cell lymphomas is focused at the 5' end of Ig joining (J_H) segments and was shown to have extra-nucleotide "N" segments at the site of the 14;18 juncture. This indicates that the immunoglobulin recombinase mediates breakage on chromosome 14 and suggests that the t(14;18) occurs early in development at a pre B cell stage. A cosmid clone of the germline region of 18q21 was obtained and a 2.8 kb major breakpoint region (mbr) where over 70% of translocations occur was sequenced and analyzed. It too can serve as a molecular marker for the translocation, and thus a transformation event enabling investigators to follow the natural history of these neoplasms.

A single translocation is invariant over time in each lymphoma. However, variations in Ig gene arrangement on the normal chromosome 14 and in light chain genes occur, and represent clonal progression rather than true biclonality. The 18q21 mbr has also been used to define lymphoma cytogenetics. Many 14q+ lymphomas in which the reciprocal partner was indeterminant by routine cytogenetics were shown to be t(14;18) by gene rearrangement. In addition, some J_H rearrangements within T cell neoplasms can be classic 14;18 translocations, indicating that B cell type translocations can spill over into T cells. Further characterization of a previously identified kappa chain gene deleting element (kde) that eliminates κ genes prior to λ light chain gene expression has been carried out.

A major new project has involved the development of techniques and reagents for use in gene therapy in the treatment of severe combined immune deficiency (SCID), which is caused by deficiency of the purine salvage enzyme adenosine deaminase (ADA). T-cell lines have been established from ADA deficient patients using HTLV-I. These T-cell lines are sensitive to deoxyadenosine and are deficient in ADA. A mouse retrovirus vector gene transfer system has been modified to contain the gene for human ADA and this vector has been tested for its ability to transfer a functional ADA gene into those ADA(-) T cells. The data show that such a gene vector system can successfully transfer a functional human gene into defective cells with high efficiency, and that such "gene"-treated cells are reconstituted to normal function in vitro.

Preliminary results from studies in monkeys have demonstrated that one can successfully induce the production of some human DNA in an ADA vector treated monkeys, again demonstrating the potential for this mode of gene transfer.

Another research project has focused on trying to understand and ultimately manipulate the immune response at an antigen-specific level. The approach has been to study the mechanisms of immune response (Ir) gene function and T cell activation by antigen in association with major histocompatibility (MHC) antigens. In previous studies it was shown that immunodominance of a site depends both on factors intrinsic to the antigens, such as helical amphipathicity and on factors extrinsic to the antigen, especially the MHC antigens of the responding individual, and thus its Ir genes. Recent studies have shown that the majority of known immunodominant sites recognized by helper T cells have a common property, namely that if they fold into a helix, that helix would be amphipathic. A computer algorithm has been developed to predict such sites from the protein sequence alone. This algorithm correctly identified 18 of 23 known immunodominant T cell sites on 12 proteins. The ability to predict T cell antigenic sites is being applied to the development of synthetic vaccines, to the AIDS virus and the malaria parasite.

Helper T cell clones, on stimulation with antigen and presenting cells, induce IL-1 secretion by macrophages. This induction is mediated by a new low-molecular weight lymphokine secreted by the T cell clones, which is now being characterized. The IL-2 receptor system plays a key role in the T-cell immune response. In the past, IL-2 binding was reported to be restricted to the 55 kd Tac peptide. Recently is has been shown that the IL-2 receptor is a complex receptor with multiple peptides in addition to the one identified by anti-Tac. The receptor gene contains 8 exons and is located in the short arm of chromosome 10.

Transcription of receptor mRNA is initiated at two discrete start sites in normal activated T cells. The 5' flanking region of the receptor gene contains a regulatory region which activates IL-2 receptor expression. This region has been cloned and sequenced. Preliminary data also suggest the presence of an enhancer-like sequence located 5' to the two promoter segments. A secreted form of the IL-2 receptor protein has been produced using an "anchor-minus" cDNA construct expressed in mouse L cells. The full-length IL-2 receptor cDNA has also been expressed in human CEM T cells. The infected CEM T cells were proven to express both high and low affinity forms of the IL-2 receptor.

The mechanism of HTLV-I- and HTLV-II-induced T-cell transformation and accompanying deregulated expression of IL-2 receptors is being explored. Utilizing retroviral vectors, the transactivator (tat) gene of HTLV-II was introduced into Jurkat T cells in both the sense and antisense orientation. These cells were then studied for altered expression of various cellular genes. Expression of genes encoding the IL-2 receptor, IL-2, and class II major histocompatibility antigens was activated in cells containing the sense tat gene but not in cells containing the antisense tat gene or infected with the control parental retrovirus lacking the tat gene. These data indicate that the transactivator gene of HTLV-I and -II not only augments viral replication but also alters the expression of genes which play a key role in T-cell activation and growth.

A clinical trial has been initiated to evaluate the efficacy of intravenously administered anti-Tac monoclonal antibody in the treatment of patients with the adult T cell leukemia. Three of the five patients studied with a very

rapidly progressing form of ATL had a very transient response. These therapeutic studies have been extended in vitro by examining the efficacy of Pseudomonas exotoxin conjugated to anti-Tac antibody to selectively inhibit protein synthesis and viability of Tac positive ATL lines. Clinical trials of PE anti-Tac have been initiated. Further studies have shown that ^{212}Bi-anti-Tac is a potentially effective and specific immunocytotoxic agent for the elimination of IL-2 receptor positive cells.

In addition to cellular IL-2 receptor (IL-2R), activated normal peripheral blood mononuclear cells and certain lines of T and B cell origin release a soluble form of the IL-2R into the culture media. The released soluble IL-2R is somewhat smaller (40-45kd) than the lymphocyte cell-surface IL-2R (50-55 kd), nonetheless it retains the capacity to bind IL-2. Using an enzyme-linked immunoabsorbent assay which employs two monoclonal antibodies that recognize distinct epitopes of the human IL-2R it was shown that normal individuals have measurable amounts of IL-2R in their plasma, and that certain lymphoreticular malignancies are associated with elevated plasma levels of this receptor. Thus, the presence of IL-2R in solution might be a sensitive indicator of lymphocyte activation or the presence of various tumor cells. Elevated levels of serum IL-2R have been found in patients with ATL, in patients with Hairy Cell Leukemia (HCL), (a leukemia of B-cell origin), and in some patients with other lymphoproliferative disorders. In patients with ATL and HCL, favorable clinical responses to therapy were associated with reductions in serum IL-2R levels which rose again with disease relapses. This suggests that serum levels of IL-2R may be useful in the diagnosis of various malignancies, in monitoring patients for responses to therapy, and surveillance for early relapse following therapy. It was also interesting to observe that patients being treated with recombinant IL-2 for other malignancies, had >1000 fold rises in the levels of serum IL-2R following IL-2 therapy. In a clinical study involving 80 patients with non-Hodgkins lymphoma, the level of serum IL-2R was found to be the best prognostic test for predicting survival at the time of diagnosis. Furthermore, significantly elevated levels of serum IL-2R have been found in patients with the Acquired Immune Deficiency Syndrome (AIDS), HTLV-III associated Lympadenopathy Syndrome (LAS), and the AIDS-related Complex (ARC). Subsequent studies demonstrated that mouse fibroblasts transfected with a single full length IL-2R cDNA produce not only cell-associated IL-2R but also soluble IL-2R. Thus a single cDNA can encode both the soluble and cell-associated forms of the IL-2R.

Work has continued on the biochemistry, molecular biology, and biologic importance of uromodulin, an 85 kilodalton immunosuppressive glycoprotein originally isolated from human pregnancy urine. Uromodulin is a heavily glycosylated single chain protein composed of approximately 30% carbohydrate. It has a high affinity for both recombinant human IL-1 and recombinant human tumor necrosis factor, and is a specific inhibitor of IL-1 in vitro. Recent evidence suggests that both IL-1 and TNF bind to uromodulin through exposed carbohydrate on the surface of the molecule. A partial amino acid sequence for uromodulin and a full length cDNA clone complete with an apparent leader sequence have been obtained.

The primary structures of insulin-like growth factors IGF I and II are closely related to insulin. The type I receptor binds IGF-1 better than IGF-II, recognizes insulin weakly, and is very similar to the insulin receptor. The type II receptor prefers IGF-II over IGF-I, does not recognize insulin, and is structurally quite distinct from the type I receptor. While the type I receptor

has been shown to be important for growth stimulation by IGF in some cells, the role of the type II receptor in mediating biologic responses is still undefined. Studies designed to investigate this protein are underway.

In the circulation, IGFs are associated with "binding" or "carrier" proteins. There are two size classes of carrier proteins, a M_r=40,000 protein which is the only carrier protein in fetal blood and a M_r=150,000 protein which is under growth hormone control and found in postnatal blood. Studies have been initiated to determine the structure of the 150 K binding protein.

The metabolism of proline and its metabolite pyrroline-5-carboxylate provide a mechanism for the intercompartmental, intracellular, and interorgan transfer of biological information. Recent studies have focused on the mechanisms mediating the effects of pyrroline-5-carboxylate (P5C) on cell regulation. P5C stimulates the early events in mitogenesis and acts synergistically with growth factors. The transfer of redox potential by P5C provides a metabolic interlock between amino acids and ribonucleotides, an interlock which is modulated by growth factors as an early event during mitogenesis. P5C serves as an intercellular communicator by transferring biologic information from one organ to another. Plasma levels of P5C in humans are responsive to dietary intake, thus, P5C acts as a nutritionally sensitive "hormone" to transfer redox potential. P5C reductase, the enzyme which catalyzes this transfer of redox potential, has been purified to homogeneity from human cells for the first time. On the basis of kinetics and regulatory mechanisms, it can be concluded that the enzyme functions primarily to catalyze the P5C-dependent oxidation of NADPH, thereby providing the mechanism for the metabolic interlock.

The Immunology Branch (Dr. David Sachs, Chief), conducts studies of the regulation and control of immune responses; structure and function of cell surface molecules, transplantation biology, molecular biology, and tumor immunology. A fluorescence activated cell sorter facility (FACS) is maintained and is involved integrally in many research studies within the Branch and in a large number of collaborative efforts with other NIH laboratories.

A primary research focus has been the study of the regulation and control of immune responses. One approach has been to examine the specificities involved in the selection of the T cell repetoire. Recent studies have demonstrated that the selection of L3T4+ T cells specific for composite class I + class II MHC determinants is regulated by both class I and class II MHC genes. This is the first demonstration of gene complementation between class I and class II MHC genes, and suggests a novel mechanism of T cell selection and immune response gene regulation in which T cells are selected during ontogeny on the basis of their specificity for composites of self-MHC and self-antigens.

Other studies have demonstrated that the generation of class II-restricted autotoxic T lymphocytes (CTL) is actively downregulated by class I restricted regulatory T cells. Thus, class II-restricted CTL are generated in response to defined class II antigens, but are suppressed in responses involving complex antigenic challenge.

Ia antigens are serologically demonstratable cell surface determinants involved in cell-cell interactions. The role of different Ia expressing populations in presenting antigen to cloned T cells has been investigated. The data suggest that the signals required for T cell activation may vary among T cell clones,

and that distinct Ia bearing antigen presenting cells may be competent for presentation to different T cells. In addition, the activation requirements of cloned and naive T cells have been evaluated. Specific "targeting" of the T cell receptor to accessory structures, by heteroaggregates of monoclonal antibodies directed to cell surface determinants, was capable of activating both T cell subpopulations. These studies provide a model system for further assessing the activation requirements for receptor-mediated triggering of resting and activated T cells.

Other studies have been directed toward analysis of the genetic regulation in T cell responses to staphyloccal nuclease (NASE). Cloned populations of antigen specific T cells and synthetic antigenic peptides were generated to analyze the structural features which determine antigenicity, as well as to analyze the mechanisms underlying selective association of a given antigenic determinant with a specific Ia restricting element. A consistent correlation was found between the fine specificity of a given clone and its MHC restriction specificity, suggesting 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.

The role of T cells in regulating the fine specificity of idiotype dominant responses to phosphocholine and azobenzene arsonate was studied. It was found that no absolute requirement exists for the participation of idiotype specific T helper cells in the generation of optimally idiotype dominant responses in the experimental systems studied. An assay system has been developed to study conjugate formation between cytotoxic cells and their targets, as an approach to elucidating the mechanisms of control of cellular immune responses. Antigen-independent conjugate formation appears to be an essential step which can occur independently of (and perhaps precede) antigen-specific recognition. Activated B lymphocytes significantly augment responses of resting B lymphocytes via a mechanism involving two components; one membrane-bound and one soluble.

A T cell differentiation factor (TCDF) has been identified, purified, and characterized. Recent studies have shown that TCDF plays an important role in the generation of cytotoxic lymphocytes. Studies of the selective loss of T cell immune responsiveness has been followed in HTLV-III/LAV seropositive individuals. It appears that the loss of T cell response to influenza virus, but not to major histocompatibility transplantation antigens, may be an early sign of the onset of AIDS.

Additional studies have shown that activated T cells infected in vitro with HTLV-III/LAV produce more virus than resting cells. It is postulated that the onset of symptomatic AIDS can be affected by the types of antigenic stimuli that HTLV-III/LAV-infected individuals encounter. A graft-versus-host reaction induced by class II MHC recognition results in the selective loss of one, but not all, of the helper T cell subsets. This selective loss of function mechanistically resembles the developmental stages of AIDS. It also is demonstrable in graft-versus-host examples that are associated with autoimmunity, and may serve as a model for T cell dysfunction in autoimmune diseases.

Other studies focus on understanding the structure and function of cell surface molecules and their role in T cell activation and recognition. Anti-Ly 6 antibodies as well as anti-T cell receptor antibodies can be used to activate cytotoxic T cells, and to stimulate proliferation of resting T cells. This suggests

that the Ly 6 molecule plays an important role in T cell activation. In other studies it was shown that single amino acid changes in MHC class I molecules result in profound effects on T cell recognition, indicating that the MHC molecule structure is susceptible to small perturbations which effect allo-recognition. Down-regulation of B lymphocyte differentiation occurs in response to a signal cooperatively generated by two distinct membrane receptors (FcγR and IgM), each occupied by its respective ligand. However, B lymphocytes from certain strains of autoimmune mice do not respond to this downregulatory signal. These findings suggest that FcγR plays a role in regulation of humoral immune responses.

Recent studies on the role of L3T4 accessory molecules in T cell interactions have indicated that, if the L3T4 molecule binds to determinants on the Ia molecule, those determinants must be encoded within the highly polymorphic external domains of Ia rather than the less polymorphic membrane-proximal domains of Ia. Other studies indicate that anti-Lyt2 antibodies can actively modulate the function of Lyt2$^+$ T cells.

Studies on the biochemistry of cytoplasmic granules of large granular lymphocyte tumor cells have resulted in the purification of two distinct trypsin-like enzymes which are major granule components. These studies aid in our understanding of the mechanism of NK cell cytotoxicity. Additional studies indicate that serine esterases in killer cell cytoplasmic granules play a vital functional role in the cytolytic process. These observations support the granule exocytosis model as the basic explanation for the mechanism of lymphocyte cytotoxicity.

A major focus of research activity has been directed toward understanding the structure and function of products of a major histocompatibility complex and manipulations of the immune response to these products. A large series of monoclonal antibodies to H-2 and Ia antigens have been produced and used both to study the complexity of MHC antigens and to develop anti-idiotypic reagents reactive with anti-MHC receptors. Analysis of cell surface antigens using these monoclonal antibodies has demonstrated two new H-2 products determined by genes within the D region.

A new method has been developed for inducing transplantation tolerance by reconstituting irradiated animals with mixtures of syngeneic and allogeneic bone marrow. These mixed chimeras are fully tolerant to syngeneic and allogeneic skin grafts and are fully immunocompetent. In in vito assays, nonspecific suppressor cells have been observed in the early weeks following reconstitution. These suppressor cells are not T cells, are radiation sensitive, and may be involved in the first phase of elimination of alloreactive cells.

Similar studies have been initiated using partially inbred miniature swine. Three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. Current projects include the detection and characterization of MHC recombinants. Transplants of kidneys between recombinant haplo-types have shown that selective matching of class II antigens frequently permits long term kidney graft survival across class I differences. Acceptance of vascular allografts has also induced systemic tolerance. In vitro studies of this tolerance indicate an absence of precursor cells capable of mounting a CTL response across a class I only difference. A series of monoclonal antibodies reactive with a variety of swine lymphocyte

surface antigens has been prepared. These antibodies are being used to study cell surface antigens in swine lymphocyte populations, as well as for T cell depletion in transplant studies.

Previous work demonstrated the existence of at least two distinct subpopulations of helper T cells that initiate the generation of allospecific cytotoxic lymphocyte responses. Current studies have demonstrated that $Lyt2^+$ T cells with helper function are the critical cells in initiating the rejection of class I MHC disparate skin grafts. This suggests that collaboration between phenotypically distinct T cell subsets is critical for the rejection of skin allografts expressing non-MHC antigens but is not a feature of the rejection of skin allografts expressing MHC antigens.

Efforts are being directed towards the prevention of control of graft vs. host disease. Studies of T cell depleted autologous bone marrow in rhesus monkeys showed the following: that low numbers of residual T cells could be detected, that residual T cell content correlated with early graft rejection, that recovery of the $T4^+$ subset in the reconstituted animals is prolonged whether the animals received T cell depleted or non-T cell depleted marrow, that $T8^+$ cells recover early during reconstitution, that graft rejection correlates best with the presence of $T4^+$ cells in the setting of a functional $T8^+$ subset, and that graft survival is significantly prolonged (without further immunosuppression) in animals receiving T cell depleted marrow.

The minor lymphocyte stimulating (Mls) determinants represent the only antigenic determinants (other than those encoded in the major histocompatibility complex) which are capable of stimulating T cell recognition at high frequency. A series of T cell clones specific for Mls^a, Mls^c, and Mls^d has been generated and is being used to define the polymorphism and allelism in this genetic system. Findings from these studies suggest that the Mls system not an alleic single locus system, but represents the products of at least two distinct and unlinked genes.

Additional research studies have centered on the regulation of expression and genomic organization of the class I MHC multigene family. Analysis of isolated class I genes reveals distinct regulatory mechanisms controlling their expression in vivo. Development of an in vitro model systems, such as transfection and transgenic mice, has allowed for detailed molecular analysis. Analysis of one of these genes has defined not only the promoter, but also positive and negative regulatory elements. Structural analysis of the class I MHC genes has revealed that although these genes may be differentially expressed, they share common structures. Although a variety of single nucleotide changes are observed in the exons of these genes, complex alterations are also seen, suggesting that some mechanism of gene conversion may operate to generate polymorphism in this family. Efforts to study and to clone the genes responsible for class II antigens in minature swine are underway.

Research has shown that proto-oncogenes encoding the nuclear-localized proteins, c-fos, c-myc, and c-myb, are transcriptionally regulated by signals transduced following mitogen binding to the surface of lymphocytes. Because oncogenes appear to be closely associated with tumor formation, these studies begin to define gene products that may play a role in the regulation of lymphocyte proliferation. In order to identify additional genes that are regulated in parallel with the above oncogenes, a cDNA library that is greater than ten-fold enriched for such sequences has been constructed.

When either anti-T3 or anti-Fcr are crosslinked with onoclonal anti-tumor antibodies, they cause fresh human peripheral blood and T and K cells to specifically lyse tumor targets. In in vitro studies, it was shown that human T cells coated with anti-T3 crosslinked to anti-tumor specifically lyse fresh tumor (but not normal) target cells. These studies demonstrate that such effector cells can specifically prevent tumor growth in an in vitro environment and justify further studies on the destruction of established tumors in vivo. The cellular basis for in vitro generation of activated killer cells against tumors has been partially established in a mouse model. In these studies the generation of anti-tumor cytotoxic cells is inhibited in a strain-dependent fashion by accessary cells. In addition, lymphokine activated killer cells (LAK) have been induced by culturing normal spleen cells with syngeneic macrophages and indomethacin, or by culturing normal spleen cells with exogenous IL2.

The Laboratory of Immunobiology (Dr. Tibor Borsos, Chief), conducts studies of the effector arm of the immune system, focusing its efforts on the analysis of the interaction of antibodies, antigens and complement components, and on the immunologic and genetic bases of tumor rejection in vivo.

Analysis of the dissociation of rabbit anti-methotrexate (MTX) IgG from cell bound MTX under various conditions has continued. Dissociation was quantitated with fluid phase hapten preventing reassociation of the anti-hapten antibodies. The extent of reduction of dissociability was a function of the anti-IgG antibody. Most effective were rabbit anti-b4 allotype antibodies. This suggests that the anti-IgG antibody reduced the mobility of the Fab arms of the anti-hapten antibody by locking the arms in a rigid position, and thereby reducing the chance of fluid phase hapten to compete with the cell surface hapten for the antigen binding site of the Fab arm. This would imply that a highly dissociable auto-antibody at a cell surface may induce only limited damage, while a large, non-dissociable immune complex would be more effective.

The binding and activity of C1q (the antibody recognizing subcomponent of C1) was studied. Recent findings suggest that fibronectin, a molecule that reacts with collagen, inhibits the interaction of C1q with IgG if the interaction between fibronectin and C1q occurs in the fluid phase before exposure to the IgG. It would appear that the physical form of bound C1q is different from that of free C1q and that generation of effective C1 cannot occur once C1q is bound.

Another approach focuses on the role of suramin on the generation of the complement mediated cell lesion. This process requires activation of complement components C8 and Cq, that function in a complex, multi-step process to penetrate the cell membrane and form a channel that permits the passage of low molecular weight substances. Recent studies show that suramin interferes reversibily with insertion and channel formation.

Studies of chemotaxis and inflammation have continued. Although fMet-Leu-Phe is thought to be the prototype of chemoattractants produced by bacteria, previous studies demonstrated that virtually all the chemotactic activity in filtrates of S. aureus is due to peptides other than fMetLeu-Phe. The current objective is to purify and determine the structure of the most active peptide. An IgG monoclonal antibody to human macrophage stimulating protein (MSP) was obtained last year. Subsequent purification steps revealed that the monoclonal antibody column purified MSP fraction contained at least 5 detectable proteins. It appears now that the antibody interacting with multiple plasma proteins is

monoclonal. Further studies, are underway to develop an antibody that detects only MSP. The interaction of leukocytes with the chemoattractant fMet-Leu-Phe results not only in directed movement but also in a metabolic burst that is associated with the generation of toxic oxygen radicals. The "burst" limits the magnitude of the neutrophil chemotactic response. Additional studies suggest that there is a rapid decay of an intermediate in the stimulus-response pathway, which may also account for the brevity of the metabolic burst.

Progress has been made in evaluating the therapeutic efficacy of infusion of plasma absorbed with Protein A-Sepharose or inactivated CNBr Sepharose in the treatment of rats with primary mammary cancer. These results provide evidence for a role of proteins of the alternative pathway of complement in the inhibition of tumor growth.

The mechanisms by which tumors recur at sites of injection of retrovirus-infected fibrosarcoma cell lines are being investigated. Previously it was shown that tumor recurrences reflect outgrowth of rare cells that lack viral antigens and are susceptible to superinfection with the homologous retrovirus. In a recent study, clones isolated from a retrovirus-infected cell line were evaluated as precursors for tumor recurrence. One clone that contained a single abbreviated copy of the provirus formed variants that lacked the proviral gene, and these tumor variants grew progressively in both nonimmune and virus-immune animals. This model may be useful in analyzing gene deletion as a mechanism of tumor escape from host immunological attack.

In a second example of this phenomenon, mouse melanoma cells were transfected with a gene ($H2^d$) encoding a foreign cell surface antigen. Cloned tumor cells expressing $H2^d$ were injected into immunodeficient mice, normal nonimmune mice, or mice immunized to $H2^d$ antigens. Pulmonary metastases from both immune and nonimmune mice (but not immunodeficient mice) had deleted the $H2^d$ gene.

The Laboratory of Cell Biology (Dr. Lloyd Law, Chief), conducts studies on the growth and repression of neoplasms. Major emphasis is placed on the study of class I (restricted) and class II (cross-reacting) tumor antigens of the transplantation rejection type (TATA). Studies of the purification and characterization of tumor specific transplantation antigens have continued. Two distinct TATAs have now been purified to chemical hemogeneity from Meth A and CI-4, both chemically-induced murine sarcomas; a 82 kD antigen and on 86 kD andigen. The purified Meth A protein retains its unique immunogenecity and specificity by in vivo testing in syngeneic mice. The NH_2- and COOH-termini analyses of the Meth A 82 kD protein indicate it to be a pure protein consisting of a single polypeptide chain, with little sequence homology to other known proteins.

Two tumor-specific antigens of 82 kD and 86 kD molecular weights have been isolated and purified to chemical homogeneity from a newly arisen sarcoma, C11-7. In in vivo assays, both were found to be highly immunogenic and tumor specific. The 86 kD (from CII-7) exists in two isoforms of 86 kD and 84 kD weights and is specific for CII-7 in vivo immunogenic assays. NH_2 terminal sequences are now being obtained for the CII-7 sarcoma 86 kD protein to determine its relationship to the Meth A protein which is now known to have sequence homology with several heat shock proteins. Further analyses should permit identification of the molecular change in this antigen which elicit cell-mediated immunity.

The main histocompatibility Class I antigens are a highly polymorphic set of integral cell surface glycoproteins involved in many aspects of the immune response. They are composed of a heavy chain which has three external domains, (N, C, and C2), a transmembrane region, and a cytoplasmic tail. Site-directed mutagenesis can be employed to study structure-function relationships of Class I histocompatibility antigens. The third external domain of class I antigens has a higly conserved disulfide bridge. To elucidate the functional significance of this disulfide bridge, a mutant H-2L^d gene has been produced in which the codon for cysteine-203 is changed to a codon for serine. Subsequent experiments suggest that the structural integrity of the third external domain is important for intracellular transport of class I antigens. Using similar techniques, it has been shown that the primary role of carbohydrates of class I antigens is to facilitate the intracellular transport of the nascent proteins to the plasma membrane.

Studies on the recognition of lysozymes by T cells have continued. The use of synthetic peptides has permitted the delineation of the fine antigenic specificity of hen egg-white lysozyme (HEL) specific suppressor- and helper- T cells. A cDNA library from a rearranged T cell suppressor clone specific for HEL has been generated and α and β T cell receptor clones have been isolated. These clones will enable the continued investigation of the role of these genes in the mechanism of T cell suppression. DNA sequences predicted to have aberrant helices with inserted bases have been crystallized. These structures may serve as a model indicating why certain sequences have a much greater tendency to mutate than others and how proteins recognize particular regions of DNA.

In continuing studies of the immunogenicity of melanoma, it has been shown that the chemically induced JB/RH and JB/MS melanomas, the ultraviolet light induced K1735 melanoma, and the spontaneous B16 melanoma, produce tumor specific transplantation antigens (TSTA) which not only induce autologous tumor rejection, but share determinants with the heterologous melanoma cell lines as well. Specificity studies have shown that the distribution of these antigens is restricted to the melanoma cells. No crossreactivity with any non-melanoma tumor lines has been demonstrable. One melanoma tumor specific antigen has been purified to homogeneity and partially characterized. It is a glycoprotein of 65 kilodaltons, which has biochemical and immunological homology to serum albumin. It is effective in immunizing mice against subsequent challenge with melanoma in transplantation assays, and demonstrates the potential immunological significance of tumor specific antigen expression on the progress of tumor growth. It has been found that alteration of surface urokinase activity significantly affects the metastatic potential of melanoms cells, suggesting that this protease plays an important role in the metastatic sequence. Monoclonal antibodies specific for the melanocyte specific enzyme, tyrosinase have been produced and are being used to examine cellular control mechanisms functional in the response of melanocytes to varying environmental stimuli which affect pigmentation, such as melanocyte stimulating hormone.

The Laboratory of Tumor Immunology and Biology (Dr. Jeffrey Schlom, Chief), carries out laboratory investigations of the biochemical mechanisms of oncogene expression and cell growth; the identification and characterization of cellular genes associated with the development of murine and human mammary neoplasia; and the development and utilization of monoclonal antibodies directed against tumor associated antigens.

A major research project currently in progress is the elucidation of biochemical events involved in regulation of cell growth whose derangements, as a consequence of oncogene expression, may cause or contribute to neoplastic cell transformation. Alterations in the synthesis of specific cellular proteins as a consequence of expression of retroviral oncogenes have been studied in murine (NIH/3T3) cells.

Studies have been designed to ascertain which, if any, of the components of the transformed phenotype are directly caused by suppression of tropomyosin synthesis. The data have established that tropomyosin suppression results from reduced levels of specific mRNAs. A cDNA library derived from NIH/3T3 mRNA was produced in the lambda-gt11 expression vector. Specific cDNA clones were selected for the various species of tropomyosin. Thirty TM4-positive plaques have been identified and purified. The synthesis and content of tropomosins and other cytoskeletal proteins in various human tumor cell lines and tissues are being investigated to determine whether synthesis of these proteins is suppressed in human neoplasms.

Studies have continued on the unique phosphoprotein, pp17, which undergoes rapid phosphorylation in HL60 promyelocytic leukemia cells in response to treatment with phorbol ester (TPA). The nonphosphorylated precursor (p17) of pp17 has been identified. p17 was found to be a major cytosolic protein in HL60. Phosphorylation of p17 is one of the most rapid, and quantitatively significant biochemical responses to TPA treatment. The possible role of p17 and pp17 in the cessation of cell growth and the onset of differentiation is being explored.

Studies on revertant cell lines resistant to transformation by the retrovial oncogene ras have been extended. Recent data indicate that the acquisition of at least 2 different patterns of potassium transport could be associated with the revertant phenotype. The possibility that alterations in monovalent cation transport is primarily responsible for the ras-induced transformed phenotype is is under study.

Additional techniques have been developed to study the effects of ouabain on various normal and transformed cells. Initial findings suggest that some human tumor cell lines are more susceptible to ouabain than other such lines, but the significance of this finding has yet to be brought to light.

Additional studies of neoplastic transformation focus on the identification of important cellular control mechanisms which affect growth and differentiation. Protein kinase C (C-kinase) appears to be active when bound to the plasma membrane of cells, and relatively inactive when present in cytosolic fractions. Membrane binding is enhanced by the tumor promoter TPA. Using a newly developed reconstitution system, it has been demonstrated that chelator-stable binding of a TPA-C-kinase complex to the plasma membrane requires calcium and phospholipids, and is regulated by calcium levels in intact cells.

The role of retinoids and hormones in mediating cell growth and differentiation is a topic of ongoing activity. 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 cyclic AMP-dependent protein kinase (cAMP-PK) activities. Recent data suggest that cellular retinoic acid binding protein (cRABP), may be required to mediate the RA-induced increase in cAMP-PK activities. Other studies have demonstrated that human psoriatic fibroblasts have

little, if any, R_{II} regulatory subunits, compared to R_{II} levels in normal fibroblasts. These changes in R_I correlate with the severity of the disease, and indicate that determination of R_I levels in red blood cells may be used as an index to establish the presence and severity of psoriasis. Human EC (Tera 2) cells in culture have insulin-like growth factor I (IGF-I) receptors present on the cell surface. Exposure of these cells to RA leads to differentiation to a neuronal-like cell type which exhibits enhanced calcitonin stimulation, and somatostatin inhibition, of aenylate cyclase activity. These findings suggest a possible role for IGF-I, calcitonin, and somatostatin during embryonic development.

Studies have shown that treatment of intact cells with tumor promoters (TPA), as well as with certain growth factors and hormones, causes a rapid redistribution or stabilization (activation) of protein kinase C to the particulate fraction. Immunohistochemical localization studies support the observation that TPA treatment mediates association of PK-C to the nucleus and cytoskeleton of NIH/3T3, with HL-60 cells. The different subcellular distributions of PK-C induced by TPA with different cell types may help to explain the different responses noted with various cell types with exposure to TPA. Further studies support the hypothesis that PK-C activation (membrane association) plays a role in regulating cell proliferation, in tumor promotion and malignant transformation, and possibly, in the modulation of this membrane signal transmission system by ras p21 protein.

Considerable research activity has focused on characterizing the genetically transmitted (endogenous) mouse mammary tumor virus (MMTV) genomes in inbred and feral strains of mice, and their potential role in tumorigenesis; on identifying the cellular genes at risk in mammary tumors induced by infectious MMTV; and on characterizing MMTV related sequences in human cellular DNA.

BALB/c mice express a unique 1.7 kbp species of MMTV RNA in the lactating mammary gland. In previous studies the chromosomal locations of the endogenous MMTV genomes (designated MTV-6, MTV-8, and MTV-9), were established and sublines containing only one of these genomes were derived. Only the MTV-6 subline expresses the 1.7 kb MMTV RNA in lactating mammary tissue. A common integration region (int-3) was identified for MMTV in MMTV induced mammary tumors. Recombinant clones of this region of the cellular genome have been obtained, and were used in the genetic analysis of the int-3 locus.

A novel family of human endogenous retroviral genomes has been described which consists of a mosaic of sequences related to different classes of infectious retroviruses, and is unrelated to mammalian type C retroviruses. Studies are in progress to determine the genetic alterations associated with breast carcinomas. Results have shown a significant correlation between rare alleles of c-H-ras-1 and breast cancer patients. The frequent loss of a c-H-ras-1 allele correlates with histopathological grade III tumors, loss of estrogen and/or progesterone receptors, and development of distal metastasis. Amplification or rearrangement of c-myc correlates with patients over 51 years of age.

Galactosyltransferases, a subgroup of the transferases, constitute a family of enzymes which transfer galactose from UDP-gal to the non-reducing residues of oligosaccharides of various glycoconjugates as well as to monosaccharides. Molecular gentic studies have been initiated in an attempt to unravel the mechanism involved in the galactoslytransferase activity which is essential

for generating specific cell-surface antigenic determinants. Sequence analyses of the cDNA clones from several sets of galactosyltransferases have shown that the regions of the mRNA coding for either carboxylterminal or other domains of the protein are identical with other cDNA clones which may code for proteins that are structurally (and may be functionally) related to other galactosyltransferases.

An area of continuing emphasis is 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 monoclonal antibodies (MAbs) have been utilized with immunohistochemical methods for the detection of occult carcinoma in surgical and cytology preparations, the phenotyping of malignant cell populations, the differentiation of histologic tumor types, and the identification of various cellular products.

Monoclonal antibodies (MAbs) of predefined specificity have been generated by utilizing a synthetic peptide reflecting amino acid positions 10-17 of the Hu-ras p21 gene product (RAP) as an immunogen. These MAbs clearly defined enhanced ras p^{21} expression in the majority of human colon and mammary carcinomas tested. Invasive mammary carcinomas demonstrated enhanced ras p21 expression with generally decreasing expression in carcinoma in situ, atypical hyperplasia, and nonatypical hyperplasia. Normal mammary and colonic epithelia, and tissues from patients with fibroadenoma and fibrocystic disease were negative. RAP MAbs have been used to define ras p21 protein expression in a spectrum of colonic disease states. The data suggest that ras p21 expression is correlated with the depth of carcinoma within the bowel wall, and is probably a relatively late event in colon carcinogenesis. Enhanced ras p21 expression was also observed in high grade prostate and bladder carcinomas.

Experiments are being conducted to determine the distribution and role of alpha transforming growth factors (TGFs) in normal and malignant rodent and human mammary epithelial cells. Transformed mouse mammary epithelial cells become resistant to the growth promoting effects of EGF because these cells have an increased capacity to synthesize αTGF mRNA. Human breast cancer cell lines are also producing αTGF and possess αTGF mRNA. There appears to be a strong positive correlation between the presence of αTGF mRNA and the presence of functional estrogen receptors in a subset of primary human breast carcinomas.

Other research projects have focused on the role of hormones and growth factors in normal mammary gland development and differentiation and in tumorigenesis. One study focuses on elucidating the nature of lactogenic hormone receptors and the factors (including other hormones) which affect binding of the hormone to this molecule. The receptor has been purified from human tissue and is being characterized.

Within human breast and colon carcinoma lesions (and in established human tumor cell lines) there is extensive heterogeneity in the expression of defined tumor antigens as recognized by the binding of monoclonal antibodies (MAbs). Studies were conducted to identify those biological response modifiers that can override the intrinsic changes in antigen expression that lead to an increase in the cell surface binding of monoclonal antibodies. Recombinant human leukocyte interferon (Hu-IFN- A) increases the binding of specific MAbs to the surface of human breast and colon carcinoma cells in a dose-dependent manner. Recent analysis revealed that this increase in tumor antigen expression is a result of an in-

creased amount of antigen per cell as well as the recruitment of previously antigen negative cells to become antigen positive. These studies may not only provide a better definition of the transformed phenotype but may enable the development of new strategies to enhance the in vivo binding of monoclonal antibodies to tumor antigens.

Monoclonal antibodies B6.2 and B72.3 bind to human breast and colon tumor associated antigens. IgG from both these monoclonal antibodies has been purified and F(ab')2 and Fab' fragments were prepared from the B6.2 IgG. The radiolabeled $F(ab')_2$ fragment of B6.2 IgG localized specifically in the breast tumor xenografts with the IgG giving maximal activity in the tumor. The F(ab')2 fragment gave the best tumor-to-normal tissue ratios due to its rapid clearance from the blood stream. Radiolabeled B72.3 localized in colon carcinoma xenografts in animal studies. Model systems that resemble the metastatic nature of human colon carcinomas are being developed to better determine the efficacy of radiolabeled monoclonal antibodies as potential agents for radioimmunodetection and radioimmunotherapy.

Clinical trials have been initiated at the NIH Clinical Center using radiolabeled Mab B72.3 to detect and localize colorectal carcinoma lesions. Various parameters will be systematically investigated concerning both the efficiency of MAb localization and the efficiency of gamma scanning of carcinoma lesions.

In previous work it was shown that cAMP antagonizes estrogen action and produces growth arrest of hormone-dependent mammary tumors; and, that cAMP and estrogen antagonism regulate the c-ras p21 oncoprotein expression in rat mammary tumors. In the present studies, the role of cAMP in growth control is being investigated employing new site-selective cAMP analogs. These studies demonstrated for the first time that the site-selective cAMP analogs are potent growth inhibitors of several breast and colon human cancer cells in culture as well as rat tumors in vivo. This suggests that the site-selective cAMP analogs may be useful in therapy of breast and colon cancers in humans. Current hypotheses concerning the escape of tumors from normal growth controls center on the observed production of growth stimulating and inhibiting factors by tumor cells. Growth factor and inhibitor production by normal and neoplastic human mammary cells in culture and in freshly isolated tissues is being examined. Four growth factors have been purified from these sources including MDGFI, MDGFII, transforming growth factor alpha ($TGF\alpha$), and transforming growth factorβ ($TGF\beta$). These factors are being purified and further analyzed to assess their role in the control of cell growth.

SUMMARY STATEMENT

LABORATORY OF MOLECULAR BIOLOGY

DCBD, NCI

OCTOBER 1, 1985 TO SEPTEMBER 30, 1986

Genes, how they work and how they can be manipulated to prevent or cure disease are topics of major interest in the Laboratory of Molecular Biology.

Gene Regulation:

One of the impediments to successful cancer treatment is drug resistance. To understand the basis of the resistance to some commonly used agents, adriamycin, vinblastine, and actinomycin D, M. M. Gottesman, I. Pastan and colleagues have developed highly multidrug resistant human cancer cells and used these cells to isolate a human gene encoding multidrug resistance (mdr-1). Using cDNAs for the mdr-1 gene, mdr-1 expression has been measured in normal human tissues and tumors and high levels of expression have been found in pheochromocytomas and normal human adrenals, colon carcinomas and normal colon, and a few cancers resistant to drug treatment. These findings suggest that measurement of mdr-1 RNA in cancers may be useful in designing treatments for different types of cancer. The product of the mdr-1 gene is a 170 kd membrane glycoprotein that binds vinblastine and relates drugs and functions to keep the intracellular concentration of these anticancer drugs low.

Other drugs that overcome drug resistance (verapamil, diltiazem, quinidine) act by preventing the anticancer drugs from binding to the membrane glycoprotein encoded by mdr-1. M. Willingham has used sophisticated fluorescence techniques to measure the uptake of daunomycin by single cancer cells and shown that multidrug resistant cancer cells accumulate less adriamycin than drug sensitive cells due to enhanced drug extrusion from the cell. This morphologic assay provides a rapid and convenient method of evaluating drugs that interfere with this efflux, and that would be potentially useful as adjunctive agents for chemotherapy.

To understand how oncogenes regulate cell growth, I. Pastan and G. Merlino have cloned the epidermal growth factor receptor gene and its promoter region. The promoter has several interesting features. One is that it resembles the promoters of SV40 virus and the human H-ras gene. Both SV40 and H-ras are oncogenic agents. This similarity suggests that expression of certain growth control genes may be regulated by a common mechanism.

B. de Crombrugghe and colleagues, Gene Regulation Section, are studying fibroblasts transformed by a series of oncogenes such as v-mos, v-ras or v-src. These cells show an inhibition of type I collagen and fibronectin synthesis and their transcripts. This system is used to study how such oncogenes disrupt the differentiation program of cells. Several cis-acting elements in the promoter of the mouse $\alpha_2(I)$ collagen gene and trans-acting factors present in nuclear extracts of normal fibroblasts that interact with these cis-acting elements have

been identified. The activity of two of these factors is reduced in fibroblasts transformed by v-mos and v-ras, whereas the activity of another factor is unchanged.

Michael Gottesman and colleagues (Molecular Cell Genetics Section) are studying the mechanism of the regulation of the growth of cancer cells using a number of different biochemical and genetic systems. Cancer cell growth can be inhibited by cAMP, and cAMP-resistant Chinese hamster ovary cell mutants have been isolated with altered cAMP dependent protein kinases. The gene encoding a mutant kinase regulatory subunit from one of these mutants has been isolated for use as a dominant moveable genetic element which can inactivate cAMP dependent protein kinase in a recipient cell. cAMP dependent expression of certain eukaryotic promoters has been shown to depend on the presence of an intact cAMP dependent protein kinase system. Temperature-sensitive mutants with an altered beta-tubulin have been studied using gene transfer techniques, and the gene encoding a mutant beta-tubulin has been identified. The major excreted protein (MEP) of transformed mouse cells has been shown to be an acid-activable thiol protease. Levels of synthesis and secretion of this protease are regulated at the level of transcription by many different oncogenes, tumor promoters and several growth factors.

B. Howard and colleagues (Molecular Genetics Section) are studying growth regulation in mammalian cells. The investigation of growth inhibitory activity detected in a DNA-mediated gene transfer system is emphasized within this framework. Molecular cloning of growth inhibitory sequences is being pursued by analysis of a cosmid library derived from WI38 human embryo fibroblast genomic DNA. A single candidate cosmid clone exhibiting high growth inhibitory function has been identified. It contains sequences that hybridize under stringent conditions to a small transcript tentatively identified as 7 SL RNA. Interestingly, this transcript is present in higher levels in WI38 fibroblasts and in HeLa cells transfected with WI38 DNA sequences and selected in the growth inhibition assay, than in parental HeLa cells. To more effectively investigate the action(s) of these growth inhibitory sequences and those of growth stimulatory genes, a new magnetic affinity cell sorting method has been developed. Applications of this method, in particular for the introduction of growth inhibitory and/or growth stimulatory genes, the anti-sense complements of these genes, or regulatory sequences flanking these genes, are being explored.

S.-y. Cheng and colleagues have identified a novel thyroid hormone binding protein present in the nuclear envelope and endoplasmic reticulum of many cell types. The protein (p55) has been purified to near homogeneity, a partial amino acid sequence determined, monoclonal antibodies prepared, and a full length cDNA isolated. Expression of this cDNA should allow determination of the role of p55 in thyroid hormone action.

G. Johnson has been studying thermal stress of cultured mouse mammary carcinoma cells which results in a loss of basal and glucocorticoid-induced mouse mammary tumor virus (MMTV) RNA within about 60 min. Cycloheximide treatment prior to the temperature shift prevents loss of the RNA. A nuclease which is subject to a rapid turnover may be activated by the heat shock.

S. Adhya and colleagues (Developmental Genetics Section) continue to study how the gal operon of E. coli is negatively regulated. gal has been shown to be repressed by two repressor molecules binding at two operators, one on either side of the promoter region. Evidence from repressor-DNA binding experiments and by altering the distance between the two operators suggests that the two repressors interact to form a DNA loop between them. The promoter is then in a conformation unfavorable for transcription. Sankar Adhya and Susan Garges continue their genetic study of how the allosteric change in the cyclic AMP receptor protein of E. coli is brought about by cAMP. By isolating, characterizing, and sequencing crp mutants that can function without cAMP, they have developed a model in which specific amino acids are involved in the allosteric change. To further study this, they have isolated crp mutants which cannot undergo the allosteric change, and starting with the cAMP-independent crp mutants, have isolated additional mutants which now require cAMP. The rho gene of E. coli, from previous in vivo work, appears to be regulated by CRP. This effect appears to be indirect and a genetic search is underway to identify the factor(s) involved.

S. Gottesman and colleagues (Biochemical Genetics Section) have been studying the role that protein degradation plays in regulating cell growth control through the study of mutants defective in ATP-dependent protein degradation. E. coli lon mutants are defective in cell division regulation after DNA damage, and this defect is due to stabilization of a highly unstable cell division inhibitor, the product of the SulA gene. lon mutants also overproduce capsular polysaccharide. Three regulatory genes have been identified which participate in the regulation of cps (capsule synthesis) genes. The protein products of two positive regulatory loci, rcsA and rcsB, have been identified. RcsA is unstable, and its turnover may be mediated by the Lon protease; RcsB is stable. The in vitro degradation of the Lon substrates, lambda N protein, insulin B chain, and glucagon, was used to define the sites of Lon cleavage. Studies on cells devoid of Lon activity demonstrate the existence of other ATP-dependent proteolysis systems in E. coli. A genetic selection for mutants with increased activity for other proteases has been developed, by selecting for mutants resistant to the unstable cell division inhibitor, SulA. Mutant candidates which are resistant to high levels of SulA apparently increase proteolysis. Using a biochemical screen for the ATP-dependent degradation of casein, a new, multi-component ATP-dependent protease in cells devoid of Lon actvity has also been identified.

S. Wickner has been studying biochemical mechanisms involved in DNA replication. Using an in vitro system that replicated supercoiled plasmid DNA containing the λ origin of replication to study the process of initiation, she has shown the reaction requires two phage initiation proteins O and P, and many E. coli proteins including DnaB, DnaG, primase, pol III holoenzyme, DnaJ, DnaK, DNA gyrase, DNA ligase and SSB, RNA transcription in the region of the origin, and a specific site, ori λ on the DNA. By deletion analysis, she has determined the minimal DNA site required for initiation. By complementaton assays, she has purified DnaJ and K proteins and is characterizing them biochemically. DnaK has weak ATPase activity, DnaJ is a DNA binding protein and both form protein complexes with λ P protein.

I. Pastan and M. Willingham and D. FitzGerald have continued in their efforts to develop immunotoxins for the treatment of adult T-cell leukemia (ATL) and ovarian cancer. In collaboration with T. Waldmann (Metabolism Branch), two patients with ATL have received a conjugate of Pseudomonas exotoxin A (PE) with an antibody to the interleukin 2 receptor (anti-TAC). This study showed that small amounts of PE-anti-TAC could be given safely to patients and defined toxic side effects that need to be overcome. PE and ricin A chain containing immunotoxins against human ovarian cancer have been shown to be active in an animal model in which human ovarian cancer cells grow in the peritoneal cavity of mice. Some of these immunotoxins are now being evaluated for possible clinical use.

M. Willingham (Ultrastructural Cytochemistry Section) has used immunocytochemistry to evaluate monoclonal antibodies for use in constructing immunotoxins specific for human ovarian carcinomas. These studies identified normal tissues reactive with antibodies to the human transferrin and IL-2 receptors, as well as with a series of monoclonal antibodies generated to human ovarian carcinoma cells (OVB antibodies). Using immunocytochemistry, he has determined the intracellular location of a thyroid hormone binding protein, p55, and another cellular protein, p86.

K. Yamada and coworkers in the Membrane Biochemistry Section have been characterizing the interactions of cells with fibronectin and collagen, with particular emphasis on the mechanisms of cell adhesion and their relevance to cell migration and metastasis. The regions of fibronectin required for binding to cells were defined. The sequence Gly-Arg-Gly-Asp-Ser plus an additional determinant were required for full binding activity. A synthetic peptide containing this pentapeptide inhibited cell adhesion *in vitro* and metastasis of B16 melanoma cells *in vivo*. The inhibition was non-toxic, dose-dependent, and specific and was due to inhibiting retention of tumor cells in the lung. Membrane-associated receptors for fibronectin and collagen were characterized. Fibronectin binding and adhesion required a 140K protein complex and a second protein of 45K. Gangliosides were implicated in fibronectin fibril formation, but not adhesion. Collagen modulated fibronectin's function, and a major protein involved in binding collagen was found to be a 47K phosphoprotein. Levels of this protein were decreased after malignant transformation, but its phosphorylation was substantially elevated. This protein is a novel, major heat shock-regulated protein. These studies have identified complex systems for mediating cell adhesion.

S. Wollman, Chief of Cell Organization Section, has retired after 37 years at NIH. He has had a distinguished career in thyriod research for which he has received numerous honors. Dr. Wollman continues as a Scientist Emeritus.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08000-16 LMB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 charactars or lass. Title must fit on one line between the borders.)*
Regulation of Gene Activity

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

PI: I. Pastan Chief LMB, NCI

COOPERATING UNITS *(if any)*
The University of Oklahoma
Institute of Virology, Tokyo, Japan
Institute of Physical and Chemical Research, Hirosawa, Wako-shi, Saitama, Japan

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

The promoter region of the EGF receptor gene contains SP1 binding sites which probably regulate its expression. A number of other possible regulatory sites have been detected in exonuclease protection assays. A full length cDNA clone of the EGF receptor gene has been constructed and used to make sense and antisense RNA. The sense RNA is translated into EGFR protein. Antisense RNA specifically inhibits this translation. Mutants of KB cells with low levels of EGF receptor have been isolated and are being characterized.

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

Other Professional Personnel:

G. Merlino	Staff Fellow	LMB, NCI
M. M. Gottesman	Chief, MCGS	LMB, NCI
S.-y. Cheng	Research Chemist	LMB, NCI
M. C. Willingham	Chief, UCS	LMB, NCI
A. Johnson	Guest Researcher	LMB, NCI
Q. Gong	Visiting Fellow	LMB, NCI
L. Beguinot	Visiting Fellow	LMB, NCI
Y. Jinno	Visiting Fellow	LMB, NCI
S. Ishii	Institute of Physical and Chemical Research, Japan	
B. Roe	University of Oklahoma	
T. Yamamoto	Institute of Virology, Tokyo University, Japan	

Project Description

Objectives:

To understand the molecular basis of cancer and growth control.

Methods Employed:

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

Major Findings:

The promoter region of the EGF receptor has been isolated and sequenced and the important structural features identified. The promoter contains five repeats of a CCGCCC sequence which represent binding sites for transcription factor Sp1. Deletion of this region of the promoter results in loss of promoter activity. Using exonuclease protection methods, a number of other important regions in the promoter have been identified which bind proteins which may be involved in transcription. A full-length cDNA encoding the EGF receptor has been isolated and used to prepare messenger RNA which is faithfully translated into EGF receptor protein. Anti-sense messenger RNAs have been prepared which specifically inhibit translation of sense EGF receptor mRNA. These anti-sense RNAs are active when injected into human cells. Epidermal growth factor has been found to stimulate synthesis of its own receptor and increase the rate of receptor protein synthesis five- to ten-fold. Stimulation is detected about two hours after EGF addition indicating it is one of the earliest effects on gene activity produced by EGF.

Mutants of KB cells with low numbers of EGF receptor have been isolated by selection in a single step with epidermal growth factor coupled to pseudomonas toxin. These mutants have low levels of epidermal growth factor receptor, grow slowly, and have cross-resistance to other toxins. The nature of

the mutations accounting for diminished EGF receptor expression is currently under study. In collaboration with Dr. Sheue-yann Cheng a thyroid hormone binding protein has been isolated, antibodies prepared, and a full length cDNA clone identified which encodes this protein. The cDNA is being characterized and sequenced to help determine the role of this protein in thyroid hormone action.

Proposed Course:

To continue to investigate the structure and function of the EGF receptor gene and its promoter. To help determine if the EGF receptor is responsible for the abnormal growth of some tumor cells. To study the role of the EGF receptor in development. To identify the function of the thyroid hormone binding protein.

Publications:

Merlino, G.T., Xu, Y.-h., Richert, N., Ishii, S., Clark, A.J.L., Stratton, R., Wilson, R.K., Ma, D.P., Roe, B.A., and Pastan, I.: Cloning and characterization of human epidermal growth factor receptor gene sequences in A431 carcinoma cells. In Feramisco, J., Ozanne, B., and Stiles, C. (Eds.): Cancer Cells. Cold Spring Harbor, New York, Cold Spring Harbor Press, 1985, pp. 19-24.

Akiyama, T., Yamada, Y., Ogawara, H., Richert, N., Pastan, I., Yamamoto, T., Kasuga, M.: Site-specific antibodies to the erbB oncogene product immunoprecipitate epidermal growth factor receptor. BBRC 123: 797-802, 1984.

Perrotti, N., Taylor, S.-i., Richert, N.D., Rapp, U.R., Pastan, I., and Roth, J.: Immunoprecipitation of insulin receptors from cultured human lymphocytes (IM-9 cells) by antibodies to pp60^{src}. Science 227: 761-763, 1985.

Ishii, S., Xu, Y.-h., Stratton, R.H., Roe, B.A., Merlino. G.T., and Pastan, I.: Characterization and sequence of the promoter region of the human epidermal growth factor receptor g;ene. Proc. Natl. Acad. Sci. USA 82: 4920-4924, 1985.

Merlino, G.T., Ishii, S., Whang-Peng, J., Knutsen, T., Xu, Y.-h., Clark, A.J., Stratton, R.H., Wilson, R.K., Ma, D.P., Roe, B.A., Hunts, J.H., Shimizu, N., and Pastan, I.: Structure and localization of the genes encoding the aberrant and normal epidermal growth factor receptor RNAs from A431 human carcinoma cells. Mol. Cell. Biol. 5: 1722-1734, 1985.

Hunts, U., Ueda, M., Ozawza, S., Abe, O., Pastan, I., and Shimizu, N.: Hyperproduction and gene amplification of the epidermal growth factor receptor in squamous cell carcinoma. Jpn. J. Cancer Res. 76: 663-666, 1985.

Tyagi, J.S., Hirano, H., and Pastan, I.: Modulation of fibronectin gene activity in chick embryo fibroblasts transformed by a temperature-sensitive strain (ts68) of Rous sarcoma virus. Nucl. Acids Res. 13: 8275-8284, 1985.

Ishii, S., Merlino, G.T., and Pastan, I.: Promoter region of the human Harvey ras protooncogene: structural similarity to the EGF receptor proto-oncogene promoter. Science 230: 1378-1381, 1985.

King, C.R., Kraus, M.H., Williams, L.T., Merlino, G.T., Pastan, I.H., and Aaronson, S.A.: Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant sized mRNAs. Nucl. Acids Res. 13: 8477-8486, 1985.

Hasumura, S., Kitagawa, S., Pastan, I., and Cheng, S.-y.: Solubilization and characterization of a membrnae 3, 3', 5-triiodo-L-thyronine binding protein from rat pituitary tumor GH_3 cells. BBRC 133: 837-843, 1985.

Clark, A.J.L., Ishii, S. Xu, Y.-h., Merlino, G.T., and Pastan, I.: Epidermal growth factor, phorbol ester and serum factors stimulate the expresion of the hum,an epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA 82: 8374-8378, 1985.

Beguinot, L. Werth, D., Ito, S., Richert, N., Willingham, M.C. and Pastan, I.: Functional studies on the EGF receptor with an antibody that recognizes the intracellular portion of the receptor. J. Biol. Chem. 261: 15938-15945, 1985.

Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamou, S., and Shimuzu, N.: High incidence of amplification of the EGF receptor gene in human squamous carcinoma cell lines. Cancer Res. 46: 414-416, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08001-15 LMB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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	LMB, NCI
Other:	M. M. Gottesman	Chief, MCGS	LMB, NCI
	D. Lowy	Chief	LCO, NCI
	A. Levitzki	Fogarty Scholar-in-Residence	LMB, 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:
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.)*

In light of the recent relationship between the ras genes and cyclic AMP synthesis in yeast, we have systematically characterized alterations in adenylate cyclase activity of NIH 3T3 cells transformed by ras genes. We have found that ras transformation produces an alteration in the ability of adenylate cyclase to respond to prostaglandin E1. We have been unable to detect any other changes in the activity of the adenylate cyclase system caused by overproduction of normal ras or mutant ras gene products. These findings suggest the the G protein encoded for by ras is not itself a component of the adenylate cyclase system in NIH 3T3 cells.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

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

Methods Employed:

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

Major Findings:

In light of the recent relationship between the ras genes and cyclic AMP synthesis in yeast, we have systematically characterized alterations in adenylate cyclase activity of NIH 3T3 cells transformed by ras genes. We have found that ras transformation produces an alteration in the ability of adenylate cyclase to respond to prostaglandin E1. We have been unable to detect any other changes in the activity of the adenylate cyclase system caused by overproduction of normal ras or mutant ras gene products. These findings suggest that the G protein encoded for by ras is not itself a component of the adenylate cyclase system in NIH 3T3 cells.

In collaboration with Michael Gottesman, we have continued to characterize protein kinase mutants of Chinese hamster ovary cells.

Proposed Course:

To examine the relationship between cyclic AMP and transformation.

Publications:

Singh, T.J., Hochman, J., Verna, R., Chapman, M., Abraham, I., Pastan, I., and Gottesman, M.M.: Characterization of a cyclic AMP-resistant Chinese hamster ovary cell mutant containing both wild-type and mutant species of type I regulatory subunit of cyclic AMP-dependent protein kinase. J. Biol. Chem. 260: 13927-13933, 1985.

Levitzki, A., Rudick J., Pastan, I., Vass, W.C., and Lowy, D.R.: Adenylate cyclase actvity of NIH 3T3 cells morphologically transformed by ras genes. FEBS Letters 197: 134-138, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08712-11 LMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Plasma Membrane Proteins in the Regulation of Cell Behavior and Drug Resistance

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

PI:	I. Pastan	Chief	LMB, NCI
Other:	M. M. Gottesman	Chief, MCGS	LMB, NCI
	M. Willingham	Chief, UCS	LMB, NCI
	N. Richert	Senior Investigator	LMB, 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:
5.5	4.5	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.)*

Human KB cells which are resistant to adriamycin (A), vinblastine (V), colchicine (C) and actinomycin D (AcD) have been shown to contain an amplified gene (mdr-1) responsible for the drug resistance. A fragment of this gene has been cloned and used to screen a cDNA library and identify cDNAs encoding a 170 kdalton membrane protein also found to be increased in multidrug resistant cells. The 170 kd protein binds vinblastine and related drugs, and this binding is overcome by drugs such as verapamil, quinidine, and diltiazem which overcome multidrug resistance. The levels of expression of the mdr-1 gene are being measured in human cancers.

PHS 6040 (Rev. 1/84)

GPO 914-918

Other Professional Personnel:

A. Fojo	Staff Fellow	LMB, NCI
V. Chaudhary	Visiting Fellow	LMB, NCI
J. Hwang	Visiting Fellow	LMB, NCI
M. Cornwell	Visiting Fellow	LMB, NCI
I. Roninson	Center for Genetics, University of Illinois College of Medicine, Chicago, IL	

Project Description

Major Findings:

The plasma membrane senses the external environment and initiates complex cellular responses. Large molecules bind to receptors and are internalized by the pathway of receptor mediated endocytosis. We have been focusing on determining where in the cell decisions are made to direct internalized ligands to their final destination and have found it to be in a tubular network on the TRANS face of the Golgi (TR Golgi). Clathrin-coated regions of the TR Golgi concentrate EGF and the EGF receptor just prior to their delivery to lysosomes.

Cell lines resistant to chemotherapeutic agents such as adriamycin (A) vinblastine (V), actinomycin D (AD) and colchicine (C) are able to protect themselves by keeping the intracellular levels of these compounds low. The plasma membrane excludes these drugs by pumping them out. We have developed multidrug resistant human KB carcinoma cells, showed that these cells fail to accumulate A, V, AD, and C, and found they contain an amplified gene (MDR-1) that encodes a 4.5 kb mRNA. Expression of the gene correlates with levels of drug resistance. When the human mdr-1 gene was transferred into mouse cells, they became multidrug resistant strongly suggesting that mdr-1 is responsible for the drug resistance. The product of the mdr-1 gene appears to be a 170K dalton plasma membrane protein. This protein appears to be a drug binding protein because: (1) Vesicles enriched in this protein bind increased amounts of vinblastine and vincristine. (2) This binding is specifically inhibited by adriamycin and verapamil (a drug which overcomes multidrug resistance). (3) A radioactive vinblastine affinity analogue specifically labels a 170K dalton protein in membrane vesicles from mdr cells. (4) Affinity-labeling of the 170K dalton protein is blocked by V, A, and verapamil and compounds related to verapamil.

A full-length cDNA encoded by the mdr-1 gene has been isolated and sequencing is almost complete. This cDNA has enabled us to measure mdr-1 expression in normal human tissues and many tumors.

Our findings provide one plausible biochemical mechanisms for the development of multidrug resistance and will provide tools to predict whether certain types of chemotherapy can be successful.

Publications:

Fojo, A.T., Whang-Peng, J., Gotttesman, M.M., and Pastan, I.: Amplification of DNA sequences in human multidrug-resistant KB carcinoma cells. Proc. Natl. Acad. Sci. USA 82: 7661-7665, 1985.

Seth. P., FitzGerald, D., Willingham, M.C., and Pastan, I.: Pathway of adenovirus entry into cells. In Notkins, A.L. and Oldstone, M.B.A. (Eds.): Concepts in Viral Pathogenesis, Vol II, New York, Springer Rolag, 1986, pp. 191-195.

Cornwell, M.M., Gottesman, M.M. and Pastan, I.: Increased vinblastine binding of membrane vesicles from multidrug resistant KB cells. J. Biol. Chem. 261: 7921-7928, 1986

Roninson, I.B., Chin, J.E., Choi. K., Gros, P., Housman, D.E., Fojo, A., Shen, D.-w., Gottesman, M.M., and Pastan, I.: Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. Proc. Natl. Acad. Sci. USA 1986 (in press).

Cornwell, M.M., Safa, A.R., Felsted, R.L., Gottesman, M.M., and Pastan, I.: Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150,000-170,000 dalton protein detected by photoaffinity labeling. Proc. Natl. Acad. Sci. USA 83: 3847-3850, 1986.

Shen, D.-w., Cardarelli, C., Hwang, J., Richert, N., Ishii, S., Pastan, I., and Gottesman, M.M.: Multiply drug resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin or vinblastine show changes in expression of specific proteins. J. Biol. Chem. 261: 7762-7770, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08753-04 LMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Immunotoxin Therapy of Cancer Cells

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

PI:	I. Pastan	Chief, Laboratory of Molecular Biology	LMB, NCI
Other:	M. C. Willingham	Chief, UCS	LMB, NCI
	D. FitzGerald	Staff Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

Metabolism Branch, DCBD, NCI
Laboratory of Theoretical Biology, Medicine Branch, DT, NCI
Cetus Corporation, Emeryville, CA

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.9	4.1	1.8

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.)*

Antibodies reacting with human ovarian cancer cells have been coupled to Pseudomonas toxin. Such immunotoxins kill ovarian cells in tissue culture and human ovarian cancer cells growing in the peritoneal cavity of nude mice. A number of monoclonal antibodies reacting with human ovarian cancer cells have been isolated by the hybridoma technique. Phase I studies have been begun in collaboration with T. Waldmann to treat adult T-cell leukemia with an antibody to the IL2 receptor conjugated to Pseudomonas exotoxin.

PHS 6040 (Rev. 1/84)

GPO 914-918

Other Professional Personnel:

R. Pirker	Visiting Fellow	LMB, NCI
J. Hwang	Guest Researcher	LMB, NCI
P. Seth	Visiting Fellow	LMB, NCI
T. Waldmann	Metabolism Branch	DCBD, NCI
R. Ozols	Medicine Branch	NCI
T. Hamilton	Medicine Branch	NCI
M. Bjorn	Cetus Corporation, Emeryville, CA	
R. Blumenthal	Laboratory Theroretical Biology	DCBD, NCI

Project Description

Objectives:

To develop methods of selectively killing human cancer cells. To determine the biochemical basis of this effect and to prepare reagents for treating human cancer.

Methods Employed:

Preparation of toxins, antibodies and toxin-antibody conjugates; growth and purification of adenovirus; chemical modification of toxins and virus, protein purification and cell culture, testing of immunotoxins in nude mice bearing human tumors; preparation of monoclonal antibodies by cell-fusion techniques.

Major Findings:

Having previously shown that PE-anti-Tac could be safely given to monkeys in dose up to 1 mg/three kilo monkey, we began a phase one study in collaboration with Dr. Thomas Waldman. Two patients with adult T-cell leukemia have been treated. Patient one received a total of 4 mg of PE-anti-Tac over two weeks and had an initial lowering of his leukemic white blood cell count without significant side effects. Patient two received 4 mg PE-anti-Tac in less than 48 hours and developed some rise in liver enzymes indicating some damage to the liver. Current efforts are being devoted to producing an immunotoxin with fewer side effects and a higher therapeutic index.

We have evaluated the activity of immunotoxins in a nude mouse model of human ovarian cancer. In this model, 60 million ovarian cancer cells are injected into the peritoneal cavity and immunotoxin treatment begun on day five or six when the tumor is well established. Significant prolongation of the survival of tumor bearing mice was produced by: (1) A pseudomonas exotoxin conjugate to a human antitransferrin receptor antibody using four doses at 0.3 µg/dose. (2) Ricin A conjugated to an antibody to the human transferrin receptor using four doses at three micrograms per dose or more. (3) Ricin A conjugated to a monoclonal antibody supplied by Cetus Corporation. These experiments indicate that immunotoxins can be active against ovarian cancer in a mouse model and suggest immunotoxin therapy might be useful if a suitable monoclonal antibody can be identified.

Balb/c mice have been immunized with human ovarian caricnoma cells. A number of hybridoma clones have been identified producing antibodies to human ovarian cancer cells and these antibodies are currently being characterized for possible use as immunotoxins.

Proposed Course:

1. To prepare anti-Tac conjugated to modified pseudomonas exotoxin; to continue trials against adult T-cell leukemia.

2. To prepare immunotoxins using newly isolated new antibodies against ovarian carcinoma cells.

3. To prepare a large panel of antibodies against human ovarian cancers.

Publications:

Seth, P., Willingham, M.C. and Pastan, I.: Adenovirus-dependent release of ^{51}Cr from KB cells at an acidic pH. J. Biol. Chem. 254: 14350-14353, 1984.

Pirker, R., FitzGerald, D.J.P., Hamilton, t.C., Ozols, R.F., Willingham, M., and Pastan, I.L Antitransferrin-receptor antibody linked to Pseudomonas exotoxin as a model immunotoxin in human ovarian carcinoma cell lines. Cancer Res. 45: 751-757, 1985.

Akiyama, S.., Seth, P., Pirker, R., FitzGerald, D., Gottesman, M.M., and Pastan, I.: Potentiation of cytotoxic activity of antibody-toxin conjugates on cultured human cells. Cancer. Res. 45: 1005-1007, 1 [illegible]

Seth, P., Pastan, I., and Willingham, M.C.: Adenovirus-dependent increase in cell membrane permeability. J. Biol. Chem. 261: 9598-9602, 1985.

Pirker, R., FitzGerald, D.J.P., Hamilton, T.C., Ozols, R.F., Bjorn, M.J., Frankel, A.E., Willingham, M.C., and Pastan, I.: Characterization of immunotoxins active against ovarian cancer cell lines. J. Clin. Inves. 76: 1261-1267, 1985.

Seth, P., FitzGerald, D., Willingham, M.C., and Pastan, I.: Pathway of adenovirus entry into cells. In Notkins, A.L. and Oldstone, M.B.A. (Eds.): Concepts in Viral Pathogenesis, Vol II. New York, Springer-Verlag, 1985, pp. 191-195.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08709-11 LMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

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

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

PI: G. S. Johnson Research Chemist LMB, NCI

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:
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.)*

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

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

Thermal stress of cultured mouse mammary carcinoma cells results in a loss of basal and glucocorticoid-induced mouse mammary tumor virus (MMTV) RNA within about 60 min. Cycloheximide treatment prior to the temperature shift prevents loss of the RNA. A nuclease which is subject to a rapid turnover may be activated by the heat shock.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

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

Methods Employed:

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

Major Findings:

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

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

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

Heat shock of cultured cells has been shown to increase poly ADP-ribosylation of proteins. This fact, coupled with the observed effects of ADP-ribosylation inhibitors on MMTV RNA described above, prompted us to explore the effect of heat shock on MMTV RNA as a possible means to learn more about regulation of this gene. We observed that a heat shock from 37°C to 42°C resulted in a loss of basal and glucocorticoid-stimulated cytoplasmic MMTV RNA; the RNA was almost non-detectable in 60 min. Treatment with cycloheximide prior to heat shock prevented the fall in MMTV RNA. The results suggest that heat shock activates a nuclease which is normally subject to rapid turnover at 37°C. Pretreatment with inhibitors of ADP-ribosylation to lower the amount of endogenous ADP-ribose did not prevent the fall in MMTV RNA. Therefore, this heat shock response may not be related to ADP-ribosylation of proteins.

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

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

Significance to Cancer Research and the Program of the Institute:

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

Proposed Course:

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

(A) Gene Expression

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

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

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

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

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

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

(B) Differentiation

(1) One approach to the treatment of cancers is the use of agents which induce maturation of cancer cells into well differentiated or benign non-malignant cells. Retinoic acid treatment of some tumors is being used in this context. Our results, in an experimental model system for human cancer, demonstrate that nicotinamide and its derivatives cause maturation of cancer cells. Moreover, their actions are synergistic with retinoic acid. Current studies are under way to evaluate the possible use of nicotinamide and N'-methylnicotinamide as therapeutic agents. This possibility is intriguing in that nicotinamide is a natural vitamin and is less toxic than are other agents, even at quite large doses.

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

Publications:

Tanuma, S.-i. and Johnson, G.S.: ADP-ribosylation and glucocorticoid-regulated gene expression. In Althaus, F.R., Hilz, H., and Shall S. (Eds.): ADP-Ribosylation of Proteins. Berlin, Heidelberg, Springer-Verlag, 1985, pp. 379-387.

Johnson, G.S. and Tanuma, S.-i.: ADP-ribose: A novel aspect of steroid action. In Althaus, F.R., Hilz, H., and Shall S. (Eds.): ADP-Ribosylation of Proteins. Berlin, Heidelberg, Springer-Verlag, 1985, pp. 372-378.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08705-10 LMB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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 professionel personnel below the Principal Investigetor.) (Name, title, laboratory, end institute affiliation)*

PI:	M. M. Gottesman	Chief, Molecular Cell Genetics Section	LMB, NCI
Other:	G. Vlahakis	Research Biologist	LMB, NCI
	M. Chapman	Research Biologist	LMB, NCI
	R. Fleischmann	Staff Fellow	LMB, NCI
	R. Kuriyama	Guest Researcher	LMB, NCI
	C. Whitfield	Guest Researcher	LMB, NCI

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:
2.5	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 spece 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. We have isolated a variety of different mutants which express mutated α- or β-tubulin subunits. These mutants are defective in spindle formation because the mutant tubulins are present in spindles and interfere with their normal function. The mutant tubulins are not spindle-specific, however, and are found in interphase microtubules. One β-tubulin mutation has been transferred by DNA mediated gene transfer to wild-type cells where it is amplified, overexpressed and renders the cells dependent on Colcemid for growth at 37°C. The mechanism of cAMP action on CHO cells has been studied by isolating CHO mutants resistant to growth inhibiton by cAMP. These mutants have defective cAMP dependent protein kinases with either altered regulatory (RI) or catalytic (C) subunits. We have used DNA from cells carrying dominant cAMP-resistant defects to transfer the cAMP-resistance phenotype to sensitive cells and have cloned segments of RI and C from cosmid libraries prepared from our mutant cell lines. The intent of these studies is to isolate cloned mutant cAMP dependent protein kinase subunits for use as movable genetic elements capable of inactivating the protein kinase system in a variety of differentiated cells and transgenic animals. We have also used the CHO protein kinase mutants to demonstrate that the expression of many promoters linked to the reporter gene encoding chloramphenicol acetyl transferase is positively regulated by cAMP in a manner which depends on cAMP dependent protein kinase activity.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

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

Methods Employed:

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

Major Findings:

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

(2) Our β-tubulin mutants and transferents are resistant to the drug DCBT (dicholorbenzylthiocyanate), proving that this drug exerts its toxic effects on cells by interacting with microtubules. Multidrug-resistant CHO and human KB mutants are sensitive to this drug, suggesting that DCBT or an analog of DCBT might be useful as a chemotherapeutic agent. DCBT produces an unusual pattern of microtubule rearrangement and its inhibition of microtubule depolymerization requires pre-incubation.

(3) We have conducted studies on the biophysical properties of the cell membranes of CHO cells and CHO tubulin mutants exposed to microtubule depolymerizing agents such as vincristine and colcemid. These agents affect membrane fluidity as measured with spin label probes of the membrane and spin-label probes of sulfhydryl membrane proteins and alter membrane potential as measured with the fluorescent dye dihexyloxacarbocyanine iodide. These effects are blocked in tubulin mutants, indicating that they are mediated through the microtubule system.

(4) We have isolated over 30 independent cAMP-resistant CHO mutants. Since most of these mutants carry dominant mutations encoding resistance to cAMP, it has been possible to transfer the cAMP-resistance phenotype from several mutants to sensitive cells via DNA mediated gene transfer. These transfers have been achieved using a two-step protocol. In the first step, genomic DNA is mixed with $_{p}SV_{2}neo$ DNA and cotransferents are selected using the neomycin analog G418. In the second step, these G418 resistant cells are selected for cAMP-resistance. Two of these transferents from different cAMP-resistant mutants have been shown to express the biochemical phenotype of the original mutants. In one case, using a cDNA probe for the regulatory subunit (R) of cAMP dependent protein kinase (obtained from S. McKnight), it has been possible to demonstrate DNA-mediated transfer of a mutant R-subunit gene.

(5) 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) and a C subunit probe (gift of R. Maurer), we have isolated cosmid clones from these libraries for the RI and C genes. These are being tested for the presence of complete coding sequences for these subunits.

(6) 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.

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

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

Proposed Course:

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

resistant and cAMP-resistant mutants will be cloned from cosmid libraries. Cloned dominant mutant genes can be used as movable genetic elements to study the function of tubulin and cAMP dependent protein kinase in a variety of differentiated cells.

Publications:

Singh, T.J., Hockman, J., Verna, R., Chapman, M., Abraham, I., Pastan, I., and Gottesman, M.M: Characterization of a cyclic AMP resistant Chinese hamster ovary cell mutant containing both wild-type and mutant species of type I regulatory subunit of cyclic AMP-dependent protein kinase. J. Biol. Chem. 260: 13927-13933, 1985.

Abraham, I., Brill, S., Hyde, J., Fleischmann, R., Chapman, M., and Gottesman, M.M.: DNA-mediated gene transfer of a mutant regulatory subunit of cAMP-dependent protein kinase. J. Biol. Chem. 260: 13934-13940, 1985.

Whitfield, C. Abraham, I., Ascherman, D., and Gottesman, M.M.: Transfer and amplification of a mutant β-tubulin gene results in colcemid dependence: Use of the transformant to demonstrate regulation of β-tubulin subunit levels by protein degradation. Mol. Cell. Biol. 6: 1422-1429, 1986.

Gottesman, M.M. and Fleischmann R.: The role of cAMP in regulating tumor cell growth. In Varmus, H. and Bishop, M. (Eds.): Cancer Surveys Oxford, England, Oxford University Press, 1986 (in press).

Gottesman, M.M., Fleischmann, R., and Abraham, I.: Molecular Genetic Analysis of Cyclic AMP-dependent protein kinase. Annals of the New York Academy of Sciences 1986 (in press).

Abraham, I., Brill, J., Chapman, M., Hyde, J., and Gottesman, M.M.: DNA-mediated transfer of cAMP resistance to CHO cells. J. Cell. Physiol. 127: 89-94, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08715-08 LMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Control of Synthesis of a Transformation-Dependent Secreted Glycoprotein

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

PI:	M. M. Gottesman	Chief, Molecular Cell Genetics Section	LMB, NCI
Other:	G. Vlahakis	Research Biologist	LMB, NCI
	M. Chapman	Research Biologist	LMB, NCI
	S. Gal	Chemist	LMB, NCI
	B. Troen	Medical Staff Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

Laboratory of Experimental Pathology, DCCP, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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.)*

Cultured mouse fibroblasts which are transformed by RNA viruses, a DNA virus or a chemical agent, all secrete a 39,000 Mr-phosphoglycoprotein (major excreted protein, MEP) in large amounts. Nontransformed murine fibroblasts secrete MEP after treatment with tumor promoters such as TPA or growth factors such as PDGF. The purified protein contains mannose 6-phosphate, the lysosomal recognition marker. It is processed intracellularly in both transformed and nontransformed cells to give two specific lower molecular weight forms, the lowest of which has a predominantly lysosomal localization. The secreted form of MEP is the precursor to a lower molecular weight novel thiol protease (cathepsin) with a pH optimum of 3-5 capable of hydrolyzing a wide variety of proteins including the extracellular matrix proteins collagen, fibronectin and laminin. The specificity of peptide bond cleavage has been determined using oxidized insulin B chain. Secreted MEP can bind to the mannose 6-phosphate receptor of many cells and be endocytosed and processed intracellularly. Transformation, TPA and PDGF stimulate MEP synthesis by increasing levels of MEP specific mRNA as measured using an MEP specific cDNA probe. The mechanism of the increase in MEP mRNA levels is increased transcription as measured in nuclear run-off experiments. We have cloned a functional MEP gene from the mouse and have identified the 5' flanking region presumed to contain the MEP promoter. We are studying this system as a model of regulation of lysosomal protease synthesis, processing and secretion as it is affected by transformation and agents which mimic the transformed state, such as tumor promoters and growth factors.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To determine the function and the mechanism of regulation of the synthesis, processing and secretion of the major excreted protein of transformed murine fibroblasts.

Methods Employed:

Cell culture; radiolabeling of cell proteins, electrophoretic and chromatographic techniques; immunoprecipitation; *in vitro* translation of mRNAs; recombinant DNA technology including full-length cDNA cloning, genomic cloning from cosmid libraries, hybrid selection techniques and Southern and Northern blots.

Major Findings:

(1) MEP is a lysosomal protein since it carries the mannose 6-phosphate lysosomal recognition marker, and is processed within cells to lower molecular weight forms of 29K and 20K which are localized to lysosomes. The purified 39K secreted form of MEP is stable at neutral pH, but between pH 3 and 4 it autodigests into lower molecular weight forms which have general endoprotease activity with a pH optimum of 3-5. This cathepsin is inhibited by leupeptin and iodoacetic acid (thiol protease). It can be endocytosed by non-MEP producing cells and processed intracellularly into lower molecular weight, presumably active, forms.

(2) Activated MEP digests the extracellular matrix proteins laminin, fibronectin and collagen, as well as the serum protein BSA and a variety of intracellular proteins. The peptide bond specificity of MEP has been determined using insulin B chain as substrate, where it cleaves Glu-Ala, Leu Val, and Tyr-Thr bonds.

(3) A cloned cDNA encoding mouse MEP recognizes a 1.8 kb mRNA in nontransformed and transformed mouse cells. The level of this MEP mRNA increases 5- to 10-fold after treatment of non-transformed cells with TPA or PDGF and approximately 30- to 50-fold in transformed cells. Similar increases in MEP transcription as measured in nuclear run-off experiments are seen, accounting for the increased mRNA levels. Stimulation of MEP mRNA levels and transcription increases caused by TPA and PDGF are blocked by cycloheximide and consequently, must require new protein synthesis.

(4) Southern blot analysis using mouse MEP cDNA probes suggests that there is only one mouse gene encoding MEP and probably only one human gene.

(5) The mouse MEP gene has been cloned. It is contained on a 13 kb segment of the cosmid pcosMMEP. pcosMMEP can be transferred to non-transformed monkey CV-1 or transformed human A431 cells where the mouse form of MEP is expressed at low levels in the non-transformed cells and at high levels

in the transformed cells. The transferred MEP gene is poorly regulated by TPA or PDGF. We have mapped the 5'-flanking regions of the MEP gene to a 2.15 kb Eco RI-Bam HI fragment which is presumed to contain the MEP promoter.

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

MEP is both a marker of transformation and a sensitive indicator of the presence of tumor promoters. The induction of MEP synthesis by tumor promoters could serve as a screening test for these agents in the environment. The protease function of MEP is assumed to have an important role in tumor growth or metastasis and specific therapy aimed at neutralizing it can be used as a potential cancer therapy.

Proposed Course:

To continue to analyze the molecular mechanism underlying induction of MEP by malignant transformation, growth factors and tumor promoters by characterizing the promoter region from genomic MEP clones; to construct MEP cDNA expression vectors for transfection of non-transformed cells and in vitro mutagenesis studies; to continue to use MEP as a marker of the molecular events involved in tumor promotion and transformation; to isolate a human MEP cDNA and the human MEP gene for use as a molecular marker of human cancer.

Publications:

Gal, S. and Gottesman, M.M.: The major excreted protein of transformed fibroblasts is an activable acid protease. J. Biol. Chem. 261: 1760-1765, 1986.

Rabin, M., and Doherty, P.J., Gottesman, M.M.: The tumor promoter TPA induces a program of altered gene expression similar to that induced by PDGF and transforming oncogenes. Proc. Natl. Acad, Sci. USA 83: 357-360, 1986.

Frick, K.K., Doherty, P.J., Gottesman, M.M., and Scher, C.D.: Regulation of the transcript for a lysosomal protein: Evidence for a PDGF-modulated gene program. Mol. Cell. Biol. 5: 2582-2589, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08754-03 LMB

PERIOD COVERED

October 1, 1985, to September 30, 1986

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

Genetic Analysis of the Multiple Drug Resistance Phenotype in Tumor Cells

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

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

I. Pastan — Chief, Laboratory of Molecular Biology LMB, NCI

COOPERATING UNITS *(if any)*

Division of Cancer Therapy, NCI

LAB/BRANCH

Laboratory of Molecular Biology

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Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	6.5	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.)*

During the course of chemotherapy of human cancers, variants which are resistant to multiple drugs frequently arise. We have been investigating the genetic and biochemical basis for this multidrug resistance (MDR) of human tumor cells to chemotherapeutic agents. A model system using the cultured KB cell, a human carcinoma cell line, has been developed in which mutant cells selected independently for resistance to high levels of either colchicine, adriamycin or vinblastine have also been found to be cross-resistant to colchicine, adriamycin, vincristine, vinblastine, puromycin and actinomycin-D. Expression of MDR correlates with the presence of a 4.5 kb mRNA encoded by the mdr1 gene which was identified and cloned from highly MDR cell lines in which it is amplified. A complete cDNA coding sequence for the mdr1 gene product has been obtained. Transfer of DNA from MDR human cells to drug-sensitive mouse cells results in expression of the complete MDR phenotype and is linked to transfer and expression of the human mdr1 gene. The human mdr1 gene maps to chromosome 7. Drug-resistance in MDR cell lines results from increased energy-dependent efflux of drugs from resistant cells and correlates with increased expression of a 170,000 dalton glycoprotein on the cell surface. Membrane vesicles from MDR cells bind increased amounts of vinblastine compared to drug-sensitive cells and contain a 170,000 dalton protein which can be labeled with a photoaffinity analog of vinblastine. This labeling is blocked by verapamil, which has been shown to reverse the MDR phenotype in cultured cells. The current working hypothesis is that the 170,000 dalton vinblastine binding protein is the product of the mdr1 gene which confers drug-resistance as part of an efflux pump mechanism.

Other Professional Personnel:

N. Richert	Senior Investigator	LMB, NCI
T. Fojo	Medical Officer	LMB, NCI
D.-W. Shen	Visiting Fellow	LMB, NCI
C. Cardarelli	Research Biologist	LMB, NCI
M. Cornwell	Guest Researcher	LMB, NCI
D. Clark	Guest Researcher	LMB, NCI
K. Ueda	Visiting Fellow	LMB, NCI
M.C. Willingham	Chief, UCS	LMB, NCI

Project Description

Objectives:

To use genetic and biochemical analysis of human multidrug-resistant cell lines and tumor tissues to understand the molecular basis of resistance to chemotherapy in tumors and to apply this knowledge to diagnose and circumvent the clinical problem of multidrug-resistance in human cancer.

Methods Employed:

Somatic cell genetic analyses including tissue culture, mutant isolation, karyotyping, cell hybridization and gene transfer; standard biochemical techniques including gel electrophoresis and chromatography; and recombinant DNA technology including cDNA and genomic cloning, Southern and Northern blots, hybrid selection and translation and DNA sequencing.

Results:

(1) Through sequential, single-step selections, we have developed human KB carcinoma cell lines which are several hundred-fold more resistant to either colchicine, adriamycin or vinblastine than the parent line. These cells are all highly cross-resistant to colchicine, vincristine, vinblastine, adriamycin, puromycin and actinomycin-D. These resistances are genetically linked as shown by coordinate appearance, coordinate disappearance in the absence of the selective agent, codominance and cosegregation in somatic cell hybrids, and cotransfer by DNA mediated gene transfer.

(2) We have cloned DNA sequences from a gene, termed mdr1, whose expression correlates with extent of multidrug-resistance in all human and rodent cells studied to date. These genomic sequences for mdr1, which we amplified in highly multidrug-resistant (MDR) KB cells, were isolated by their homology to genomic probes originally described by Roninson (Nature, 309, 626, 1984) from multidrug resistant hamster cells. Identical clones were obtained from the in gel renaturation analysis of our human KB cell lines which can be used to detect and clone amplified sequences. At low levels of resistance gene amplification is not seen, but increased expression of a 4.5 kb mRNA detected by the mdr1 probe is observed. At high levels of resistance, up to

100-fold amplification of the mdr1 gene is observed. These data suggest that in the KB system low-level resistance arises by increased expression of a cellular gene, whereas high-level resistance requires its amplification.

(3) Additional evidence that the mdr1 gene is involved in the development of MDR comes from an analysis of mouse cells made MDR by transfer of DNA from human MDR cell lines. Primary and secondary transfected mouse cells contain amplified copies of the human mdr1 gene and express this human gene.

(4) Using the mdr1 probe, we have screened a cDNA library made from RNA isolated from a KB MDR cell line. A set of overlapping cDNAs covering 5 kb of expressed mdr1 sequences has been obtained and preliminary sequence data indicates that most of these represent an open reading frame.

(5) Using human mdr1 cDNA probes, we have screened a variety of normal human tissues and tumors for mdr1 gene expression. In normal tissues, expression is highest in adrenal, kidney, liver and colon. Some, but not all tumors, show increased expression of the mdr1 gene relative to normal tissue from the same patient.

(6) A set of overlapping cosmid clones containing most of the human mdr1 gene from an MDR cell line has been obtained. These cloned sequences comprise >100 kb of genomic DNA, suggesting that the mdr1 gene is large with at least several large introns.

(7) The sequential development of MDR is correlated with decreased accumulation of drugs due to decreased uptake and increased efflux by the resistant cells. This accumulation defect is reversed by treatment of cells with the Ca^{2+}-antagonist verapamil and other drugs such as quinidine which also reverse the drug-resistant phenotype of the cells. Using the fluorescent drug daunomycin, these results can be shown in single cells at the light microscopic level. These data suggest that drug resistance is due to a membrane alteration or alterations which affect drug uptake and efflux in the KB cells.

(8) High resolution two-dimensinal gel analysis of drug-resistant cell lines suggests that a small number of specific protein alterations are associated with the development of multiple drug resistance. These include the loss of a family of cell surface glycoproteins of molecular weights 70-80,000 and the appearance of a protein of molecular weight 21,000 in some cell lines. In addition, an increase in expression of a 170,000 dalton membrane glycoprotein can be demonstrated using antibodies developed by Ling and Tsuruo.

(9) Membrane vesicles from MDR cell lines bind more ^{3}H-vinblastine than membrane vesicles from drug-sensitive cell lines. A photoaffinity analog of vinblastine synthesized by A. Safa in R. Felsted's laboratory (NCI) labels a 170,000 dalton protein in these membrane preparations which comigrates with the 170,000 dalton protein found using the antibodies of Ling and Tsuruo. Binding and labeling can be blocked with other cross-resistant drugs such as vincristine and daunomycin and with verapamil which reverses the MDR phenotype. The 170,000 dalton protein is a candidate for the drug efflux pump in MDR cell lines.

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

The goal of these studies is to understand the basis of multiple drug-resistance in chemotherapy-resistant human tumors so that rational therapy can be devised to prevent or circumvent the multiple drug-resistance phenotype. Dramatic success in treatment of some human tumors with chemotherapy suggests that chemotherpay can be curative in many more cases if steps can be taken to reduce the appearance of drug resistant tumor cells. One possible approach, if a cell surface protein is responsible for multiple drug-resistance, would be to use toxin-antibody complexes to kill cells with the antigen in question on their surfaces. Another approach would be to use cloned DNA probes for drug-resistance genes to detect their expression before treatment and modify treatment modalitites accordingly.

Proposed Course:

To continue to study the mechanism of drug efflux and to develop an in vitro system to study drug accumulation in membrane vesicles derived from multidrug-resistant cells; to isolate a full-length cDNA clone for the mdr1 gene, determine its sequence and find out whether its expression in drugsensitive cells is sufficient to result in multidrug-resistance; to determine the protein product of the mdr1 gene; to continue to screen normal human tissues and human tumors to elucidate the normal function of the mdr1 gene product and whether its expression correlates with drug-resistance in clinical situations.

Publications:

Fojo, A., Akiyama, S.-i., Gottesman, M.M., and Pastan, I.: Reduced drug accumulation of multiply drug-resistant human KB carcinoma cell lines. Cancer Res. 45: 3002-3007, 1985.

Shen, D.-w., Cardarelli, C., Hwang, J., Cornwell, M., Richert, N., Ishii, S., Pastan, I., and Gottesman, M.M.: Multiple drug resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin or vinblastine show changes in expression of specific proteins. J. Biol. Chem. 261: 7762-7770, 1986

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Roninson, I.B., Chin, J.E., Choi, K., Gros, P., Housman, D.E., Fojo, A., Shen, D.-w., Gottesman, M.M., and Pastan, I.: Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. Proc. Natl. Sci. USA 1986 (in press).

Fojo, A.T., Whang-Peng, J., Gottesman, M.M., Pastan, I.: Amplification of DNA sequences in human multidrug-resistant KB carcinoma cells. Proc. Natl. Acad. Sci. USA 82: 7661-7665, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08717-08 LMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Structural Analyses and Functions of Receptors for Cell Adhesion Proteins

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

PI:	K. M. Yamada	Chief, Membrane Biochemistry Section	LMB, NCI
Other:	K. Nagata	Visiting Associate	LMB, NCI
	S. Saga	Visiting Fellow	LMB, NCI
	S. K. Akiyama	Guest Researcher	LMB, NCI
	S. S. Yamada	Guest Researcher	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

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Membrane Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.7	0.8

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Cells appear to interact with structural and regulatory molecules of the extracellular matrix by means of specific receptors. We have analyzed putative receptors for two major glycoproteins, fibronectin and collagen. A 140,000 dalton avian membrane protein complex was demonstrated to be a fibronectin receptor by three independent criteria, including binding activity, peptide and antibody-inhibition evidence, and competition for cell adhesion to fibronectin. This fibronectin receptor was localized immunologically in developing embryos. It was also found to display distinct patterns of localization depending on the migratory state of the cell. Additional modes of cell interaction with fibronectin were defined. A protein of 45,000 daltons with a pK of 5-6 was found to be involved in fibroblast adhesion to fibronectin, but not to laminin or spreading factor/ vitronectin. The process of formation of fibrils of fibronectin by cells, but not adhesion, seemed to require complex gangliosides, as shown by several approaches including the use of somatic cell variants and inhibition of fibril formation by a ganglioside-binding fragment of fibronectin. Collagen was found to modulate fibronectin-cell interactions, and a major collagen-binding protein of fibroblasts was characterized. It was shown to be a phosphoprotein of 47,000 daltons with an unusually high pK of 9.0. It was decreased after malignant transformation, accompanied by a seven-fold increase in phosphorylation. It was also found to be novel, major, heat shock-regulated protein. We propose to compare the localizations of these binding proteins in normal and transformed cells relative to each other and to intracellular cytoskeletal proteins, and to investigate their possible roles in invasion by localization and inhibition studies. We will continue biochemical characterizations of these receptors and will determine compositions, ligand specificities, and molecular organization in terms of transmembrane organization and possible cytoskeletal interaction mechanisms. Finally the regulation of these receptors will be examined in normal, transformed, and heat-stressed cells.

PHS 6040 (Rev. 1/84)

GPO 914-818

Project Description

Objectives:

(1) To identify, localize, and analyze the biological functions of cell surface receptors for fibronectin and collagen.

(2) To examine the regulation of fibronectin and collagen receptors, especially in neoplastic cells.

(3) To determine the major structural features, functional organization, and mechanisms of action of these receptors using monoclonal antibodies, proteolytic dissection, and reconstitution experiments.

Methods Employed:

Rat hybridomas were generated after injection of Sprague-Dawley rats with purified preparations of the 140k fibronectin receptor membrane protein complex or the 47k collagen-binding protein, followed by fusion of spleen cells with Y3 myeloma cells plated at cloning densities. High-affinity monoclonal antibodies were identified by screening for binding to a discrete antigen band in Western immunoblots after extensive washing. Hybridomas were adapted to grow in serum-free medium, and monoclonal antibodies were purified from culture supernatants by ammonium sulfate precipitation and DEAE chromatography. The 140k protein complex was isolated with the monoclonal antibody JG22E. Crude membrane preparations from 13 day chick embryos were solubilized with 40 mM octylglucoside, then the protein was isolated using the monoclonal antibody immobilized on agarose. It was eluted with diethylamine and dialyzed against isotonic buffer containing octylglucoside.

The interaction of the 140k complex with fibronectin was assayed using a purified 75,000 dalton cell-binding fragment of fibronectin immobilized on Sepharose beads in 0.29 x 28-cm polypropylene columns and equilibrated with column buffer consisting of 40 mM octylglucoside in PBS. In some cases, 1 mg/ml of the synthetic peptides with the sequence Gly-Arg-Gly-Asp-Ser or the sequence Gly-Arg-Gly-Glu-Ser was added to the column buffer and the 140k complex sample was preequilibrated with 1.5 mg/ml peptide.

The 140k complex was added to columns in a total volume of 90 µl either as monoclonal antibody-purified material or as a chick embryo fibroblast (CEF) extract. The CEF extract was prepared fresh daily as follows: CEF were extracted on ice for 10 min with 1 ml ice-cold extraction buffer consisting of 200 mM octylglucoside and 3 mM phenylmethylsulfonyl fluoride in PBS. The CEF extract was centrifuged 40,000 g for 20 min at 4°C, diluted to 100 mM octylglucoside and applied to the affinity column. Chromatography was performed at a flow rate of 0.9 ml/h, and 90 µl fractions were collected. Each fraction was assayed for 140k by spotting 3-µl aliquots on nitrocellulose grids prepared by drawing 1-cm squares on filter paper. The dotted

nitrocellulose sheets then were incubated in 5% (wt/vol) BSA in 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.0 overnight at room temperature or for 90 min at 37°C. The dot blots were incubated in a 1/200 dilution of polyclonal rabbit antibody to 140k in 5% BSA in Tris-saline for 90 min, then rinsed extensively. The blot was then incubated with 5 x 10^5 cpm/ml ^{125}I-labeled protein A in 5% BSA and Tris-saline for 30 min. Radioactivity was quantitated by excising each spot along the pencil lines and counting using a gamma counter.

An antibody neutralization assay was developed to identify other molecules required for cell interactions with fibronectin. Depending on the nature of the antigen preparation, either a solid-phase or a liquid-phase assay was used to measure the capacity of the preparation to neutralize polyclonal antibody preparations.

For the solid-phase neutralization assay, antigen preparations were transferred to nitrocellulose membranes or CNBr paper. Each piece of immunoblot membrane was soaked in 5% BSA overnight at 4°C or for 2 hr at room temperature, chopped with scissors into pieces <1 x 1 cm, and placed into the 1.6-cm diameter well of a 24-well dish. Each well then received 250 µl of a 250-400-µg/ml IgG solution, and the dish was agitated on a rocker for 1 to 2 hr at 4°C. After completion of the absorption, the overlying liquid was aspirated for use in biological assays, and the blot was discarded.

For the liquid-phase neutralization assay for solubilized membrane components, the detergent was removed by incubation with equal volumes of Bio-Beads SM-2 prewashed with PBS in the presence of 5% BSA in PBS for 10 min in the upper compartment of a Centrex 0.2-µm filter unit. The detergent-free solution was recovered by centrifugation at 1,000 rpm for 3 min, then mixed with concentrated IgG preparations to achieve a final IgG concentration of 250 µg/ml in 220 µl. The mixtures were incubated for 2 hr at 0°C.

IgG preparations absorbed by either of the methods described above were used in the standard cell spreading inhibition assay, and activities were compared to parallel dose-response curves using nontreated IgG, which were performed for each assay. The neutralizing activity of an antigen preparation was expressed as micrograms of antibody neutralizing units (µg-nu) calculated by the expression $(c_1 - c_2) \times V$, where the concentration and volume of the IgG solution during incubation with antigen were c_1 (µg/ml) and V (ml), respectively, and where the activity of the adsorbed IgG preparation was equivalent to the activity of a concentration of the original IgG preparation of c_2 (µg/ml).

Major Findings:

A 140,000 dalton avian membrane protein complex previously implicated in cell-fibronectin interactions was finally shown to be a fibronectin receptor by documenting its direct interaction with the cell-binding fragment of fibronectin and its sensitivity to inhibition by a synthetic fibronectin recognition-signal peptide, by showing that antibodies directed against it

blocked the binding of fibronectin to cells, and by demonstrating competitive inhibition of cell adhesion to fibronectin by an excess of the purified, exogenous 140,000 dalton protein complex. By immunofluorescence microscopy, this receptor complex was found to be present throughout the developing embryo, but was often concentrated at sites rich in fibronectin. Its distribution on individual cells was found to differ depending on the migratory state of the cell; it existed in a fibrillar pattern parallel to that of fibronectin and intracellular actin bundles in relatively stationary cells, but it converted to a diffuse pattern in rapidly migrating neural crest and somite cells.

Although the 140,000 dalton complex appears to be crucial for cell adhesive interactions with fibronectin, a second protein of approximately 45,000 daltons and pI = 5.3-6.2 was demonstrated to be involved in cell interactions with fibronectin. This glycoprotein appeared to be required for adhesion of murine cells to fibronectin, but not to laminin or spreading factor/vitronectin. Mammalian fibronectin receptor function thus appears to require more than one protein receptor component on the cell surface. The role of gangliosides in cell interactions with fibronectin was clarified by a collaborative study using ganglioside-deficient variant cells in which a role for gangliosides in primary adhesive events was ruled out. Instead, a role for these glycolipids in fibronectin fibrillogenesis was indicated in comparisons of the ability of a set of somatic cell variants in ganglioside expression to form fibrils, by enzyme and cholera-toxin blocking experiments, and by a direct demonstration that fibronectin fibril formation could be disrupted in a dose-dependent fashion by a 70,000 dalton fragment of fibronectin which lacks any cell adhsion site, but which binds to gangliosides.

Our previous demonstration that collagen can modulate cell interactions with fibronectin suggested the importance of examining cellular receptors for collagen. A membrane glycoprotein of 47,000 daltons was found to be the only major protein besides fibronectin to bind to gelatin or type I collagen under physiological salt conditions after solubilization from membranes by detergents. Like fibronectin and collagen, this collagen-binding protein was shown to be decreased substantially in transformed chick fibroblasts. This 47,000 dalton glycoprotein had an unusually high pK of 9.0 and was also found to be phosphorylated. This phosphorylation was increased 7-fold in malignant cells. Quantitites of this major collagen-binding protein were found to be substantially increased by heat shock, making it the first membrane protein of defined function to be identified as being regulated by this intensively studied mechanism of gene regulation. Preliminary work has produced monoclonal and polyclonal antibodies to this protein that should be useful for future localization studies modeled on those we performed previously for the fibronectin receptor. Preliminary results suggest localization of this 47,000 dalton collagen-binding protein in the endoplasmic reticulum and possibly at plasma membrane sites of interaction with actin.

Significance to Cancer Research and the Program of the Institute:

Invasion and metastasis are hallmarks of malignant cells, yet relatively

little is known about the biochemical mechanisms by which they interact with the molecules of the tissues they invade. Such interactions within extracellular spaces are likely to occur by plasma membrane receptors, especially those for fibronectin and collagen. Our studies are attempting to characterize the structures, regulation, and functional mechanisms of these two receptors, as well as examining how they influence each other and are altered by transformation. Information on these two receptor systems and their regulation in normal and malignant cells should further our understanding of the metastatic process.

Proposed Course of Research:

We propose to extend our studies on receptors for fibronectin and collagen with the following studies:

1. The localization of the 47,000 dalton collagen-binding protein will be determined by immunofluorescence microscopy of intact and transversely frozen-sectioned cells. Its localization will be compared with that of the fibronectin receptor and of cytoskeletal proteins thought to be involved in movement and invasion such as alpha-actinin, vinculin, actin, and talin.

2. The effects of malignant transformation on localization of the fibronectin- and collagen-binding proteins will be determined, with particular emphasis on their location relative to the rosettes or "invadopodia" characteristic of RSV-transformed cells, which contain high concentrations of oncogene product. The effects on their distribution of fibronectin-mediated reconstitution of normal morphology in transformed cells will also be examined to explore the potential regulation of fibronectin and collagen distribution by this ligand.

3. The possible roles of fibronectin and collagen receptors in invasion will be examined by localization studies, e.g., of in vivo invasive events or in vitro models for invasion, and by in vitro inhibition studies using anti-receptor antibodies and synthetic peptide inhibitors.

4. The fibronectin and collagen-binding proteins will be characterized in detail biochemically. Their specificities for various ligands will be examined in greater detail. An existing panel of 120 monoclonal antibodies to the fibronectin receptor will be used to evaluate structural features, including whether the complex is organized in transmembrane fashion. If time permits, the set of antibodies to the collagen receptor will also be expanded, and the composition, subunit organization, and amino-terminal sequence will be determined.

5. Both receptors will be examined for interactions with the cytoskeletal proteins alpha-actinin, talin, and vinculin; if specific interactions are found, attempts will be made to map the binding sites for these proteins to specific subunits or domains of the receptors.

6. Regulation of the collagen-binding protein by heat shock will be characterized initially using actinomycin D and by measuring translatable

mRNA. We will test the role of carbohydrates in its collagen-binding function using the inhibitor tunicamycin and by in vitro translation. Attempts will be made to screen a cDNA expression library with the antibodies to these receptors or, if necessary, with synthetic oligonucleotides, to obtain clones for primary sequence determinations and future functional analyses.

Publications:

Hasegawa, T., Hasegawa, E., Chen, W.-T., and Yamada, K.M.: Characterization of a membrane-associated glycoprotein complex implicated in cell adhesion to fibronectin. J. Cell. Biochem. 28: 307-318, 1985.

Yamada, K.M., Akiyama, S.K., Hasegawa, T., Hasegawa, E., Humphries, M.J., Kennedy, D.W., Nagata, K., Urushihara, H., Olden, K., and Chen, W.-T.: Recent advances in research on fibronectin and other cell attachment proteins. J. Cell. Biochem. 28: 167-193, 1985.

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Yamada, K.M., Humphries, M.J., Hasegawa, T., Hasegawa, E., Olden, K., Chen, W.-T., and Akiyama, S.K.: Fibronectin: Molecular approaches to analyzing cell interactions with the extracellular matrix. In Edelman, G.M., and Thiery, J.P. (Eds.) The Cell in Contact: Adhesions and Junctions as Morphogenetic Determinants. John Wiley and Sons, Inc., New York, 1985, pp. 303-332.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08011-12 LMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Structures and Roles of Transformation-Sensitive Cell Surface Glycoproteins

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

PI:	K. M. Yamada	Chief, Membrane Biochemistry Section	LMB, NCI
Other:	M. Obara	Visiting Fellow	LMB, NCI
	M. S. Kang	Biotechnology Training Program Fellow	LMB, NCI
	S. K. Akiyama	Guest Researcher	LMB, NCI
	M. J. Humphries	Guest Researcher	LMB, NCI
	K. Olden	Guest Researcher	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.3	3.3	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.)*

Fibronectin is a major cell surface and extracellular matrix glycoprotein that is often decreased on tumor cells, and which is involved in cell adhesion and migration. The regions of fibronectin required for interactions with cells were defined defined using a a new direct binding assay. Crucial binding information was provided by both the sequence (Gly)-Arg-Gly-Asp-Ser and by additional distant peptide information that was needed for full binding affinity; other adhesive recognition sites may also exist on fibronectin. The peptide sequence Arg-Gly-Asp-Ser, present in fibronectin, fibrinogen and von Willebrand factor, was found to be required for platelet adhesion. The possible role of such adhesive recognition signals at one or more steps in metastasis was tested using the B16 experimental metastasis system. The peptide Gly-Arg-Gly-Asp-Ser was a non-toxic inhibitor of pulmonary tumor formation. The inhibtion was dose-dependent and specific, since a conservative substitution or an amino acid transposition resulted in loss of activity. The active peptide inhibited the retention of tumor cells in the lung, suggesting an effect on early adhesive events. Our future objectives will be to complete in vitro tests of the specificity and roles of this fibronectin adhesive recognition signal in other types of cell adhesion using other adhesion molecules, and to extend our studies of peptide inhibiton of metastasis by determining specificities using a full panel of peptides, by optimizing pharmacological properties, and by examining for roles of the immune system and platelets in the inhibitory process. We will also attempt to define a hypothesized second cell-recognition signal on fibronectin with possibly distinct cell-type specificity, define further the parameters defining specificity and affinity of these peptide inhibitors using novel synthetic peptide variants, and will continue sequencing of the gene and attempting to produce genetically engineered variant polypeptides with modified or novel biological activities.

HHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

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

Our objectives are:

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

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

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

Methods Employed:

Plasma fibronectin was purifed from citrated human plasma by gelatin-Sepharose affinity chromatography and elution either under non-denaturing conditions by 50 mM sodium citrate, pH ᶜ.5, or by elution and further purification in urea using heparin-Sepharose columns. The 75,000 dalton cell-binding fragment of fibronectin was generated by controlled tryptic degradation of human fibronectin and was purified by sequential DEAE, gelatin-affinity, and heparin-affinity chromatography steps. The 11,500 dalton cell-binding fragment of fibronectin was generated by exhaustive pepsin digestion and purified by affinity chromatography using a new rat monoclonal antibody designated 333. The purity of fragments was confirmed by SDS gel electrophoresis. Synthetic peptides were synthesized according to our specifications by Peninusla Laboratories, then further purified by Fractogel TSK HW40S chromatography or reversed phase HPLC on a preparative C18 bonded silica column. Purity was assessed by reversed phase HPLC and quantitative amino acid analysis.

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

For cell adhesion assays, solutions of plasma fibronectin or other adhesion proteins were incubated with tissue culture dishes for one hour. Non-specific adsorption sites were blocked by 30 min. incubation with 10 mg/ml heat-denatured bovine serum albumin (80°C for 3 min.). Competitive inhibiton of

cell adhesion and spreading on plastic or type I collagen substrates was evaluated with purified synthetic peptides at 5-1000 μg/ml.

B16-F10 murine melanoma cells, a line selected in vivo for high lung colonization, were routinely tested for microbial infection and verified to be mycoplasma free. Peptides were solubilized to 30 mg/ml in divalent cation-free Dulbecco's phosphate-buffered saline, neutralized, and sterilized by microfiltration in 0.2 μm nylon Centrex tubes. B16-F10 cells were detached for 2 minutes with 0.02% EDTA and resuspended gently to 7×10^5/ml in Dulbecco's MEM. Peptide was then mixed with cells, and 0.2 ml aliquots containing the indicated amount of peptide or buffer with single cell suspensions of 7×10^4 cells were injected slowly into the lateral tail vein. Fourteen days later the animals were sacrificed with ether, their lungs excised, fixed in 10% formaldehyde and the number of surface melanoma colonies counted visually or with the aid of a dissecting microscope. Extrapulmonary tumor formation was checked for each group.

Major Findings:

Fibronectin is a major cell surface and extracellular glycoprotein that is considered to be a protype cell adhesion molecule involved in cell migration. We have been exploring the structural basis of its activities, focusing on the elements essential for its interaction with cells and for normal and malignant cell adhesion, migration, and invasion.

The regions of the fibronectin molecule required for interactions with cells were defined using a new assay system for quantitating the binding of fibronection or its fragments to the cell surface. Full activity was preserved in a 75,000 dalton fragment representing 15% of the intact dimeric protein, which bound to cells with an affinity of $K_d = 4 \times 10^{-7}$ M, a value at least as high as that of intact fibronectin. Further cleavage of the molecule to a fragment of 11,500 daltons resulted in a 10- to 100-fold decrease in affinity. Further reduction in size to the synthetic peptides (Gly)-Arg-Gly-Asp-Ser surprisingly retained affinities of binding similar to the 11,500 dalton fragment. These results indicate that this simple peptide recognition sequence contains virtually all of the crucial binding information within an 11,500 dalton region of the protein, but that more distal information that remains to be defined is also involved in obtaining full binding affinity.

Earlier studies on fibroblast adhesion to fibronectin were extended to an evaluation of possible adhesive recognition signals necessary for platelet aggregation and adhesion to substrates. Tests of our panel of 11 synthetic peptides related to the fibronectin adhesive recognition signal revealed a high level of inhibitory specificity for the peptide sequence Arg-Gly-Asp-Ser sequence in platelet adhesion to fibronectin, fibrinogen, and von Willebrand factor; this tetrapeptide sequence is now known to be present in each platelet adhesion protein. The mirror-image, inverted peptide Ser-Asp-Gly-Arg was inactive, as were peptides with conservative amino acid substitutions or transpositions of amino acids. Very similar results were obtained with human platelets and chicken thrombocytes. Other preliminary studies suggested that a second adhesive recognition site may exist on fibronectin that appears to

have differing peptide specificities. This putative second site appears to be recognized by certain non-fibroblastic cells of ectodermal derivation, and it differs from the other recognition site in its marked sensitivity to the synthetic peptide Gly-Arg-Gly-Glu-Ser.

We hypothesized that such adhesive recognition signals might be necessary for one or more steps in the processes of invasion and metastasis. In vivo studies of experimental metastasis revealed that fibronectin synthetic peptides such as Gly-Arg-Gly-Asp-Ser can serve as non-toxic inhibitors of B16 melanoma metastatic tumor formation in the lungs of mice. The inhibtion by peptides was dose-dependent and specific, since substitution of a Glu residue for the Asp residue caused a complete loss of activity; similarly, a transposition of two amino acids to produce the peptide Gly-Arg-Asp-Gly-Ser was also inactive, as were mixtures of individual amino acids or unrelated control peptides. The peptide did not affect cellular tumorigenicity. The site of peptide inhibiton appeared to be at an early event, perhaps inhibiting an early adhesive step, since the peptide interfered with the retention of tumor cells in the lung during the first few hours after injection. Preliminary work with additional fibronectin peptides suggests that the pattern of inhibition may resemble that of in vitro peptide inhibition of fibronectin and spreading factor, but not of laminin or native collagen.

Sequencing of genomic clones of fibronectin was continued, and preliminary characterizations of the nucleotide sequences of the cell-binding site and the promoter region of the gene were described. Preliminary work has established human fibronectin cDNA clones in phage and plasmid expression vectors to attempt the eventual production and biological assaying of the activities of polypeptides derived from truncated or mutated versions of the gene.

Significance to Cancer Research and the Program of the Institute:

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

Proposed Course of Research:

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

(1) To complete comparisons of the in vitro specificity of a variety of synthetic peptides derived from the fibronectin sequence in inhibiting the adhesion of mammalian and avian fibroblasts to fibronectin, spreading factor/

vitronectin, laminin, and type I collagen gels, and to examine their effects on cell-to-cell adhesion.

(2) To extend our studies showing inhibiton of metastasis by synthetic peptides in experimental models by (a) evaluating sequence specificity using a full set of variant synthetic peptides; (b) determining peptide clearance rates; (c) designing peptide conjugates with favorable pharmacological properties; and (d) determining the role of immune system activity and platelet-tumor cell interactions in the inhibitory action of the peptides.

(3) To test our hypothesis that a second cell-recognition site exists on fibronectin that may be cell- or tissue-specific and to attempt to define the primary sequence information required for its function.

(4) To continue the sequencing of fibronectin genomic clones, especially in possible regulatory or key functional regions, and to attempt to use recombinant DNA methods to produce novel cell adhesion polypeptides containing normal or mutated adhesive recognition signals. For example, genetically engineered fragments containing the minimal sequence necessary for full binding affinity to the cell surface might produce particularly active inhibitors of experimental metastasis. Other experiemnts could test the functions of the proposed first- and second-site adhesive signals by substituting one for the other, or by disrupting function by in vitro mutagenesis of cloned sequences.

(5) To continue testing new synthetic peptide variants of the fibronectin adhesive recognition signal to define the parameters determining binding affinity and specificity. Such studies might permit construction of non-peptide analogues of these signals. They would ideally proceed in parallel with in vitro DNA mutagenesis/expression experiments, which could evaluate longer peptide regions.

Publications:

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Akiyama, S.K., and Yamada, K.M.: Synthetic peptides competitively inhibit both direct binding to fibroblasts and functional biological assays for the purified cell-binding domain of fibronectin. J. Biol. Chem. 260: 10402-10405, 1985.

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Haverstick, D.M., Cowan, J.F., Yamada, K.M., and Santoro, S.A.: Inhibition of platelet adhesion to fibronectin, fibrinogen and von Willebrand factor substrates by a synthetic tetrapeptide derived from the cell-binding domain of fibronectin. Blood 66: 946-952, 1985.

Yamada, K.M., Akiyama, S.K., Humphries, M.J., Yamada, S.S., and Santoro, S.A.: Platelet and fibroblast interactions with fibronectin and other adhesive proteins. In Jolles, G., Legrand, Y., and Nurden, A.T. (Eds.): Biology and Pathology of the Platelet - Vessel Wall Interactions. New York, Academic Press, 1986 (in press).

Hirano, H., Kubomura, S., Obara, M., Gotoh, S., Higashi, K., and Yamada, K.M.: Structure of the chicken fibronectin gene. In International Symposium on Biology and Chemistry of Basement Membranes. Amsterdam, Elsevier Science Publisher, 1986 (in press).

Akiyama, S.K., and Yamada, K.M.: Fibronectin. Adv. Enzymol. 1986 (in press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08700-14 LMB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression of Collagen Genes in Normal and Transformed cells

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

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

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Gene Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)

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

B

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

1. A functional analysis of deletions in the promoter of the mouse α_2(I) collagen gene indicates there are two different segments upstream of the start of transcription that are important for optimal expression of this gene. In addtion, an element for negative regulation has been identified. The same negative element is present in the α_1(III) collagen promoter.
2. Transgenic mice were generated in which an α_2(I) collagen promoter-chloramphenicol acetylase chimeric gene has been stably introduced in the germline. These new mouse strains show a tissue specific pattern of expression for the chimeric gene that coincides with that of the endogenous type I collagen genes.
3. Several factors present in nuclear extracts of NIH 3T3 cells have been identified, which bind to defined segments of the α_2(I) collagen and α_1(III) collagen promoter.
4. The levels of binding activities for two factors which bind to the α_2(I) collagen promoter are decreased in nuclear extracts of NIH 3T3 cells transformed by v-mos, v-ras or treated by phorbol esters.
5. v-fos encodes a transcriptional transactivation function that is detected by cotransfection of a v-fos containing plasmid together with a tester plasmid containing the α_1(III) collagen promoter fused to the gene for chloramphenicol acetyl transferase. Cotransfections with v-fos containing plasmids lead to stimulation of the α1(III) collagen promoter. A frame-shift mutation in v-fos inhibits the transactivation. Another promoter which is activated by v-fos is the LTR promoter of Rous Sarcoma virus. Three mutants which map around -100 fail to respond to v-fos whereas other promoter mutants do respond.

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

Other Professional Personnel:

M. Mudryj	Chemist	LMB, NCI
A. Schmidt	Visiting Associate	LMB, NCI
P. Rossi	Guest Researcher	LMB, NCI
A. Hatamochi	Visiting Fellow	LMB, NCI
J. Oikarinen	Visiting Fellow	LMB, NCI
P. Golumbek	Microbiologist	LMB, NCI
B. Peterkofsky	Research Chemist	LB, NCI
B. Paterson	Research Chemist	LB, NCI

Project Description

Major Findings:

1. Functional analysis of the cloned α_2(I) promoter.
We have constructed a number of recombinant plasmids to study the activty of this promoter after DNA mediated transfection of mouse fibroblasts. In these plasmids, the α_2(I) collagen promoter has been placed at the 5' end of a marker gene. As marker genes, we have used either the bacterial gene for chloramphenicol acetyl transferase or the baterial gene which confers resistance to an analog of neomycin called G418.

A deletion analysis indicates that at least two separate segments are important for optimal expression of this promoter. One is located between -900 and -498, the other between -342 and -98. A more detailed analysis of the second segment shows that this segment also contains a negative regulatory element.

Deletion analysis of the α_1(III) collagen promoter has identified a negative regulatory element. The sequence of this element shows strong similarities with the negative element present in the α_2(I) collagen promoter.

2. Transgenic Mice.
Eight transgenic mice were generated in which the promoter of the mouse α_2(I) collagen gene (-2000 to +54) linked to the bacterial gene for chloramphenicol transacetylase is stably integrated in the germline. The copy number of the α_2(I) collagen promoter CAT unit and its site of integration in the genome vary from one strain to another. The transgenes are stably transmitted to the progeny of the different strains following a classical Mendelian segragation pattern. In seven of the eight strains, the CAT gene is expressed, although the levels of CAT enzyme activity vary considerably from one strain to the other. In six of these strains, the expression of the CAT gene follows the expected tissue distribution pattern of the α_2(I) collagen gene. Hence, the α_2(I) collagen promoter sequences present in the recombinant plasmid used for our experiments contain the necessary information to ensure tissue specific activity of this promoter.

3. Three different assays have been used to identify in nuclear extracts of NIH 3T3 cells; factors which bind to the promoter of the α_2(I) and α_1(III) collagen genes. Several such factors have been identified. One binds to a segment of the α_2(I) collagen promoter centered around the CCAAT sequence between -80 and -84. Another factor binds around -300 to a sequence which presents some homologies with a Nuclear Factor I binding site. Another factor binds to a sequence around -410. At least one other factor binds around -200.

4. The levels of DNA binding activity of three of these factors were examined in nuclear extracts of NIH 3T3 cells transfomed by either v-mos or v-ras. The levels of binding activity for two of these factors (those binding to -80 and -410) are decreased in nuclear extracts of v-mos or v-ras transformed cells, whereas the levels of the third factor (the one binding to the -300 segment) are unchanged. In v-mos and v-ras transformed cells, the synthesis of type I collagen RNA is strongly decreased. The same decrease in type I collagen RNA synthesis occurs after TPA treatment of NIH 3T3 cells. There is also a decrease in the levels of DNA binding activity in nuclear extracts of NIH 3T3 cells treated with TPA for the same two factors as were found in nuclei from v-mos transformed cells.

5. The product of the oncogene v-fos, which has a nuclear localization, displays a transcriptional transactivation function. This was shown by cotransfection of a v-fos containing plasmid together with a tester plasmid.

Publications:

de Crombrugghe, B., Schmidt, A., Liau, G., Setoyama, C., Mudryj, M., Yamada, Y., and McKeon, C.: Structural and functional analysis of the genes for α_1(I) and α_1(III) collagens. In Fleischmajer, R., Olsen, B.J., and Kuhn, K. (Eds.): Annals of the New York Academy of Sciences 460. New York, New York Academy of Sciences, 1986, pp. 154-162.

de Crombrugghe. B., Liau, G., Setoyama, C., Schmidt, A., McKeon, C., and Mudryj, M.: Structural and functional studies on the interstitital collagen genes. In Evered, D., and Whelan, J. (Eds.): Ciba Symposium on Fibrosis 114. Pitman Press, London, 1986, pp. 20-33.

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Westphal, H., Overbeek, P.A., Khillian, J.S., Chepelinsky, A.B., Schmidt, A., Mahon, K.A., Bernstein, K.E., Piatigorsky, J., and de Crombrugghe, B.: Promoter sequences of murine αA crystallin, murine α_2(I) collagen or of avian sarcoma virus genes linked to the bacterial chloramphenicol acetyl transferase gene direct tissue-specific patterns of chloramphenicol acetyl transferase expression in transgenic mice. Cold Spring Harbor Symposium on Quantitative Biology Volume L. 411-416, 1985.

Setoyama, C., Frunzio, R., Liau, G., Mudryj, M., and de Crombrugghe, B.: Transcriptional activation encoded by the v-fos gene. Proc. Natl. Acad. Sci. USA 83: 3213-3217, 1986.

Setoyama, C., Hatamochi, A., Peterkofsky, B., Prather, W., and de Crombrugghe, B.: V-fos stimulates expression of the α_1(III) collagen gene in NIH 3T3 cells. Biochem. Biophys. Res. Commun. 136: 1042-1048, 1986.

Liau, G., Szapary, D., Setoyama, C., and de Crombrugghe, B.: Restriction enzyme digestions identify discrete domains in the chromatin around the promoter of the mouse α_2(I) collagen gene. J. Biol. Chem. 1986 (in press).

Hatamochi, A., Paterson, B., and de Crombrugghe, B.: Differential binding of a CCAAT DNA binding factor to the promoters of the mouse α_2(I) and α_1(III) collagen genes. J. Biol. Chem. 1986 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08714-09 LMB

PERIOD COVERED: October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Mode of Action of a Bacterial Function Involved in Cell Growth Control

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

PI: S. Gottesman Research Chemist LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Molecular Biology

SECTION: Biochemical Genetics Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	7.0	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have been studying the role that protein degradation plays in regulating cell growth control, through the study of mutants defective in ATP-dependent protein degradation. E. coli lon mutants are defective in cell division regulation after DNA damage, and we have previously demonstrated that this defect is due to stabilization of a highly unstable cell division inhibitor, the product of the SulA gene. lon mutants also overproduce capsular polysaccharide; we have identified three regulatory genes which participate in the regulation of cps (capsule synthesis) genes. The protein products of two positive regulatory loci, rcsA and rcsB, have been identified. RcsA is unstable, and its turnover may be mediated by the Lon protease; RcsB is stable. The third regulatory locus, rcsC, serves as a negative regulator of the system, but may have additional roles as well. We have used the in vitro degradation of the Lon substrates lambda N protein, insulin B chain, and glucagon, to define the sites of Lon cleavage. While Lon has preferred sites of cleavage on these molecules, the preferred sites do not represent any simple amino acid sequence. Studies on cells devoid of Lon activity demonstrate the existence of other ATP-dependent proteolysis systems in E. coli. A genetic selection for mutants with increased activity for other proteases has been developed, by selecting for mutants resistant to the unstable cell division inhibitor, SulA. Mutant candidates which are resistant to high levels of SulA apparently increase proteolysis. Mapping studies, now in progress, should allow the identification of the proteases involved. Using a biochemical screen for the ATP-dependent degradation of casein, we have identified a new, multi-component ATP-dependent protease in cells devoid of Lon activity.

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

Other Professional Personnel:

A. Torres-Cabassa	Staff Fellow	LMB, NCI
M. Maurizi	Expert	LMB, NCI
T. Klopotowski	Visiting Scientist	LMB, NCI
K. Quinlan-Walshe	Research Microbiologist	LMB, NCI
J. Brill	Guest Researcher	LMB, NCI
J. Trempy	Postdoctoral Fellow	LMB, NCI
Y. Fujimura	Visiting Fellow	LMB, NCI

Project Description

Objectives:

We are interested in the role which proteolysis can play in regulating important cell timing functions. As an approach to this, we are investigating the effects on cell growth of an E. coli mutation in a gene coding for an ATP-dependent protease, and developing genetic and biochemical methods for studying other bacterial proteases.

Methods Employed:

Standard microbial genetic and biochemical techniques.

Major Findings:

(1) We have continued the characterization of mutations in genes involved in the regulation of capsular polysaccharide synthesis. rcsA, a positive regulator, has been shown to code for an unstable 26kd protein. The in vivo instability of the protein (five minute half-life) may be due to Lon protease; lon mutants show slower turnover of the protein. rcsA has been cloned into M13 vectors. Deletions isolated in these vectors allow the identification of the minimal size for the gene and will enable us to sequence the rcsA structural gene and regulatory region. rcsA is subject to negative regulation: multi-copy plasmids carrying defective copies of rcsA titrate the negative regulator and therefore increase the levels of expression of the chromosomal $rcsA^+$ gene. We are currently identifying the site necessary for this escape synthesis by cloning bits of the rcsA gene in multi-copy plasmids. rcsC, a known negative regulator of the system, is one candidate for the repressor of rcsA.

(2) rcsB, a second positive regulator of capsule synthesis, has been cloned from λ vectors into a variety of plasmid vectors, and the identification of a 24kd protein as the product of rcsB has been confirmed in maxicells. Insertional mutagenesis of the rcsB clone has demonstrated that mutations late in the rcsB gene produce a negatively complementing species when present on multi-copy plasmids. This suggests that RcsB normally acts as a multimer, either with itself or another component of the capsule regulation system.

(3) rcsC, a negative regulator of capsule synthesis, has also been cloned in multi-copy plasmids; the product of the gene is not easily visible in maxicells. Therefore, we have cloned the gene downstream from the regulatable plac promoter to increase transcription of the gene. In addition, we are saturating the clone with both insertion and point mutations to unravel a set of complex phenotypes we have observed in previous rcsC mutants and revertants. This will also better define the size of rcsC.

(4) Physiological studies on the natural regulation of capsule synthesis suggest that synthesis is highest when cells are grown in minimal media under aerobic conditions, at low temperature. Changing any of these factors will decrease transcription of the cps genes. We have begun the construction of appropriate cps - lac plasmid substrates to allow the in vitro identification of the steps at which these physiological controls act and the in vitro reconstruction of the roles of the rcsA, B and C regulators.

(5) The sites of cleavage of Lon protease in a number of substrates has been determined, using HPLC to separate the products of proteolysis and analyze the amino acid composition of the fragments. Insulin B chain is rapidly cleaved at a small number of sites; one primary site is cleaved initially. Lambda N protein is also cleaved at a defined number of sites, but the amino acid sequences at the cleavage sites for these two proteins do not follow any simple rule.

(6) We have begun the genetic identificaiton of proteases other than lon by developing a selection for increased activity of proteases in lon^- null cells. SulA, an inhibitor of cell division, is degraded by the Lon protease; lon^- cells cannot support plasmids carrying $sulA^+$ because the basal level of synthesis from the multi-copy plasmid is sufficient to inhibit cell division. We have mutagenized lon^- hosts and selected those which can tolerate the $sulA^+$ plasmid. Among these, we have chosen for further study those which are also resistant to the induction of the cellular copy of sulA. Two such mutants have been isolated and are being further characterized. A simple screen for SulA turnover has been developed, using SulA antibody we had previously isolated. Using this screen, we have confirmed that at least one of our candidate mutants possesses an increased ability to degrade SulA. Further analysis of this mutant should allow us to identify the mutated protease or regulator.

(7) In lon^- cells, we had previously detected a low level ATP-dependent protease activity. Further purification, using casein degradation as an assay, has demonstrated the presence of a multi-component ATP-dependent protease. We are interested in determining the role of ATP for this protease, identifying the genes responsible for the synthesis of the subunits of the protease complex, and determining the natural substrates of the protease.

Significance to Cancer Research and the Program of the Institute:

An understanding of the growth control of E. coli may serve as a model for understanding growth control in normal and transformed mammalian cells. Protein degradation is believed to play an important role in the control of

mammalian cells; insight into such processes should be gained by further investigation of the process in E. coli.

Proposed Course of Research:

Elucidate the mechanism of regulation of capsular polysaccharide synthesis, at the genetic and biochemical level. Analyze the mechanism of Lon degradation of cellular substrates and the reasons for protein instability, using Lon substrates such as N as model systems. Develop a Lon substrate with defined crystal structure to allow the identification of three-dimensional cues for Lon action. Identify and purify other energy dependent proteases in E. coli. Define mutants defective for these proteases and use the mutants to identify in vivo substrates.

Publications:

Gottesman S., Trisler, P., Torres-Cabassa, A., and Maurizi, M.R.: Regulation via proteolysis: The E. coli lon system. In Leive, L. (Ed.): Microbiology 1985. Washington, D.C., American Society for Microbiology, 1985, pp. 350-354.

Maurizi, M.R., Trisler, P. and Gottesman, S.: Insertional mutagenesis of the lon gene in Escherichia coli: lon is dispensable. J. Bacteriol. 164: 1124-1135, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08719-06 LMB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development and Uses of Eukaryotic Vectors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: B. H. Howard Chief, Molecular Genetics Section LMB, NCI

COOPERATING UNITS *(if any)*
LMV, DCCP, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Genetics 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

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 in a DNA-mediated gene transfer system is emphasized within this framework. Molecular cloning of growth inhibitory sequences is being pursued by analysis of a cosmid library derived from WI38 human embryo fibroblast genomic DNA. A single candidate cosmid clone exhibiting high growth inhibitory function has been identified. It contains sequences that hybridize under stringent conditions to a small transcript tentatively identified as 7SL RNA. Interestingly, this transcript is present in higher levels in WI38 fibroblasts, as well as in HeLa cells transfected with WI38 DNA sequences and selected in the growth inhibition assay, than in parental HeLa cells. To more effectively investigate the action(s) of these growth inhibitory sequences and those of growth stimulatory genes, we have developed a new magnetic affinity cell sorting method. Applications of this method, in particular for the introduction of growth inhibitory and/or growth stimulatory genes, the anti-sense complements of these genes, or regulatory sequences flanking these genes, are being explored.

GPO 904-917

Other Professional Personnel:

R. Padmanabhan	Chemist	LMB, NCI
N.-Z. Xu	Visiting Fellow	LMB, NCI
M. Fordis	Expert	LMB, NCI
A. Attallah	Senior Investigator	DBB, CDB
T. Howard	Guest Researcher	LMB, NCI
M. McCormick	Guest Researcher	LMB, NCI
R. B. Stead	Medical Staff Fellow	LMB, NCI
I. Pastan	Chief, Lab. of Molecular Biology	LMB, NCI
M. Willingham	Chief, Ultrastructural Biochem. Section Section	LMB, NCI
F. Blasi	Professor, International Institute of Genetics and Biophysics, Naples, Italy	
R. Reeves	Professor of Biochemistry, Washingtion State University	
R. Pozzatti	Guest Researcher	LMV, NCI
G. Khoury	Chief, Lab. of Molecular Virology	LMV, NCI

Project Description

Objectives:

The first objective of this project is development of vectors and techniques to extend the usefulness of gene transfer in the study of mammalian cell growth regulation. The second objective is the use of this technology to identify, isolate, and characterize genes and/or DNA sequences that inhibit mammalian cell growth. The third objective is analysis of mRNA and protein levels, in conjunction with gene transfer, to characterize mechanisms by which growth regulatory genes exert their effects.

Methods employed:

Vectors designed for amplification of genes in E. coli and subsequent transfer transfer to mammalian cells are crucial to this area of research. In the pSV2 family of vectors the ampicillin resistance cistron and origin of replication from the plasmid pBR322 are used for selection and propagation in E. coli; simian virus 40 ($SV4^0$) early region promoter and mRNA processing signals are used to direct expression in mammalian cells of Tn9 chloramphenicol acetyltransferase (cat), E. coli xanthine-guanine phosphoribosyltransferase (gpt), or Tn5 aminoglycoside phosphotransferase (neo) coding sequences. In the pRSV family of vectors the promoter in the long terminal repeat (LTR) of Rous sarcoma virus (RSV) is substituted for the SV40 early promoter to direct expression of cat, gpt, or neo coding sequences.

These vectors are most frequently introduced into mammalian cells using the calcium phosphate-DNA coprecipitation technique. Alternate methods involving DEAE-dextran/DNA complexes, and electrofusion are investigated where appropriate. Retrovirus vectors introduce foreign genes by infection,

with helper functions provided by coinfection or by earlier propagation in specialized non-producing helper cells.

Several assays for expression of these vectors in mammalian cells are available. When CAT vectors are used, total transient expression (12-60 hrs after transfection) may be assayed by determining ^{14}C chloramphenicol acetylated by CAT in cell extracts. With other vectors Northern or S1 analysis can be used to determine steady-state mRNA levels. The percentage of cells expressing vector DNA may be assayed by detection of intracellular CAT with rhodamine-labeled CAT antibody or CAT antibody in conjunction indirect rhodamine or peroxidase labeling. Both affinity-purified and monoclonal CAT antibodies are available. The number of cells stably expressing vector genes may be assayed by colony selection using the gpt, neo, or dhfr markers. Colonies selected may be amplified and used used for quantification of integrated exogenous DNA copies (Southern blots), mRNA (Northern and S1 nuclease analysis), or CAT activity.

Finally, to isolate mammalian DNA sequences detected by these techniques, genomic libraries are constructed using either cosmid vectors or λSV2/ cosmid vectors.

Major Findings:

I. Vector development and gene regulation studies

A. λSV and derivative vectors: λSV2 vectors, which integrate by site-specific recombination into the chromosome of the specialized λ lysogen N6106, were previously described. Uses of λSV2 or its cosmid derivatives to reconstruct integrated genes by homologous recombination are being pursued (in collaboration with Drs. George Gaitanaris and Max Gottesman; Institute of Cancer Research, Columbia University) at a low priority level.

B. Retrovirus vectors: Vectors based on mammalian retroviruses offer the possibility of extremely efficient introduction of foreign sequences into many cell types. We previously constructed a retrovirus vector that carries a mouse cDNA methotrexate-resistant dihydrofolate reductase gene, generated high titer helper virus-free stocks, and used these to infect mouse bone marrow stem cells (collaboration with G. Schwartz; AFRRI) or preimplantation mouse embryos (collaboration with A. and D. Singer; IB, NCI). Consistent with the results from multiple other laboratories, expression of retrovirus genomes in these cell types was difficult to detect. Suggestive evidence was obtained that infection of mouse bone marrow cells, followed by transplantation into irradiated mice and selection with methotrexate, resulted in increased resistance to this drug. This data has not been reproduced, however, and is of uncertain significance. Further progress in this area awaits development of retrovirus vectors that resist transcriptional inactivation and the general availability of lymphokines, especially IL-3, produced by recombinant DNA methods.

C. Vectors that code for novel cell surface proteins (collaboration with Drs. Ira Pastan and Mark Willingham): Vectors which allow physical separation of cells that are transiently expressing exogenous DNA would be extremely useful.

To this end we constructed a vector, pRSV-IL2, which directs high levels of IL-2 receptor synthesis under control of the Rous sarcoma virus long terminal repeat. We also obtained the vector pMSV-G, which directs synthesis of vesicular stomatitis virus G protein (VSV G), and monoclonal antibodies against IL-2 receptor and the VSV-G protein. A new magnetic affinity cell sorting method was de- e ped in which VSV-G coated magnetic particles are used to purify monkey kidney CV 1 cells transfected with pMSV-G. To obtain relatively high sorting efficiency and specificity, it was necesssary to devise sorting conditions under which non-specific particle/cell and cell/cell interactions are reduced to very low levels. Recent results suggest that we can enrich CV 1 cells that are transiently expressing VSV-G protein from 10-20% to >95%. There are multiple potential applications of this method, including introduction of growth inhibitory and/or growth stimulatory genes, the anti-sense complements of such genes, or their flanking regulatory sequences.

D. Gene regulation studies with CAT vectors: Putative promoter sequences from the 5' untranscribed regions of the human β-globin and ε-globin genes were cloned upstream from the CAT reporter sequence, generating the plasmids p βGLcat and p εGLcat, respectively. These plasmids were introduced into two hematopoietic cell lines, D562 and MEL, as well as several non-hematopioetic cell types (HeLa, CV-1, and WI38). Two control plasmids were used: pSV0 h h s uk yotic promoter sequences upstream from the CAT gene, and pRSVcat, which has the Rous sarcoma virus long terminal repeat. The results of these experiments indicate that: i) sufficient globin regulatory information resides in the 5' regions of these genes to restrict globin gene expression to hematopoietic cells, and ii) K562 cells contain trans-acting factors that negatively regulate β-globin expression.

II. New methods to improve DNA-mediated stable transformation efficiencies.

A. Effects of sodium butyrate: Evidence was published that treatment of transfected cells with sodium butyrate results in both increased plasmid DNase sensitivity and hyperacetylation of histones on plasmid molecules during the transient expression phase.

B. Effects of SV40 T antigen expression: It was previously observed that the frequency of stable transfection of WI3 an e sts with pRSVneo could be increased 5 - 10 fold by cotransformation w th either SV40 DNA or a non-replicating recombinant genome, pRSV-TAg, in which T antigen expression is directed by the RSV long terminal repeat. More recent evidence generalizes this result to other cell

types, e.g., CV-1 cells, and suggests that T antigen increases the fraction of cells competent to express exogenous DNA. For these studies pRSVcat-transfected cell populations were stained with affinity purified CAT antibody (provided by Dr. Ira Pastan).

C. Transfection of lymphocytes. The growth stimulation of quiescent lymphocytes by a variety of mitogens represents a well-characterized model for mammalian growth regulation. For this reason, and because lymphocytes promise to be favorable cells for our magnetic affinity cell sorting method, we were interested in developing expertise in transfecting these cells. Electroporation studies with CAT reporter vectors did not yield promising results. DEAE dextran mediated transfection, on the other hand, proved to be relatively efficient. Of particular interest is the finding that increasing the concentration of DMSO used to stimulate uptake of DEAE dextran/DNA complexes to 30% results in substantially increased CAT vector expression.

III. Application of gene transfer methods to detection of genes that regulate cell growth.

A. Malignant transformation of rat embryo fibroblasts: (collaboration with Drs. R. Pozzatti and G. Khoury; LMV, DCCP, NCI). It was previously found that primary rat embryo fibroblasts (REF) could be stably transformed (criteria: growth in soft agar and formation of tumors in nude mice) by cotransfection with the c-H-ras gene and the selectable marker pRSVneo. Genes such as adenovirus E1A and c-myc enhanced the frequency of, but were not absolutely required for, complete malignant transformation. Moreover, c-H-ras transformed REF demonstrated dramatically higher metastatic potential in a nude mouse model system than REF transformed with a combination of E1A and c-H-ras. This collaboration was completed and results of these studies published.

B. Studies relating urokinase expression to metastatic potential. Research from multiple laboratories has established a strong correlation between high urokinase (UK) expression and acquisition of the metastic phenotype. To investigate whether elevated urokinase levels are sufficient to increase metastatic potential, we placed cloned human urokinase coding sequences under control of the Rous sarcoma virus (RSV) long terminal repeat and introduced the resulting RSV-UK hybrid gene into either mouse LB6 cells or monkey kidney CV-1 cells. Clones expressing high amounts of urokinase were identified by a fibrinogen overlay assay. LB6(RSV-UK) clones tested by subcutaneous injection into nude mice exhibited increased local hemorrhage but did not metastasize more frequently than controls. Similarly, when LB6(RSV-UK) clones were tested for the capacity to establish lung metastases following injection into the tail veins of nude mice, no increased metastasis relative to controls was observed. CV-1(RSV-UK) clones were non-tumorigenic.

Since the human urokinase gene product does not recognize urokinase receptors on the surface of murine cells, the murine urokinase gene has been placed under control of the RSV long terminal repeat. Effect(s) of its expression in LB6 cells are currently being tested.

C. Inhibition of HeLa cell replication: In previous annual reports we described evidence that sequences present in deeply quiescent WI38 human embryo fibroblasts are capable of slowing HeLa cell growth. Results were obtained from experiments in which HeLa cells in monolayer culture were transfected with pRSVneo DNA in combination with either E. coli, HeLa, or WI38 genomic DNA. Forty-eight hours after transfection, cells from each culture were transferred into suspension medium containing bromodeoxyuridine and Hoechst 33258. After 48 hrs in suspension, cells were exposed to fluorescent light for 5 minutes, then plated into G-418 medium. This protocol was designed to select cells that were prevented by exogenous DNA sequences from replicating in suspension culture and, in addition, stably expressed the pRSVneo selectable marker. We determined that that transfection with WI38 DNA increased cell survival at least 10 fold more efficiently than either E. coli or HeLa S3 DNA. Moreover, the average growth rate of G-418 resistant cells following transfection with the WI3 ixture was about 2 fold slower than controls.

The major effort of this group over the past year has focused on confirming and extending these observations as follows:

i) Search for more rapid assays for growth inhibitory sequences: We are attempting to develop an assay based on cotransfection of growth inhibitory sequences with a cell surface protein expression vector, treatment with colchicine, and fluoresence activated cell sorting (FACS) to determine the fraction of transfected cells that have been fixed in the G0/G1 phase of the cell cycle. To this end the vector pRSV-IL2 was constructed and (in collaboration with Dr. J. Barker; LNP, NINCDS) tested in a FACS assay. Early results indicate a high signal-to-noise ratio; current efforts are directed at developing a double sorting assay that simultaneously scores fluorescence due to bound IL-2 receptor antibody and propidium iodide. The latter reagent allows determination of DNA content and hence position in the cell cycle. A second major project a allow more facile measurement of growth inhibitory activity is based on the magnetic affinity cell sorting method (see section IC).

ii) Genomic DNAs prepared from other sources have been examined for the presence of growth-inhibitory sequences. It has been confirmed that genomic DNA from MRC-5 cells rendered quiescent by maintenance at confluence in 0.5% fetal calf serum is strongly positive for growth inhibitory activity. In addition,

preliminary experiments indicate that genomic DNA from deeply quiescent human foreskin fibroblasts contain growth inhibitory sequences. With respect to the earlier observations that DNAs from VA-13 (a post-crisis SV40-transformed WI38 derivative), and T24 (a c-ras positive human tumor cell line), inhibit HeLa growth, it has become clear from work on immortalization mechanisms in other laboratories that these tumor cell lines belong to a different complementation group than HeLa cells.

iii) Cosmid libraries containing E. coli, HeLa, or WI38 genomic sequences were previously constructed. The WI38 library exhibited a strong growth-inhibitory signal, suggesting that clone(s) carrying functional WI38 sequences of interest were present. The E. coli and HeLa libraries were 8-20 fold less active. Fractionation of the WI38 library into sublibraries with complexities ranging from 70 to 70,000 colonies suggested that sequences with the potential to inhibit HeLa cell growth may have a copy number in excess of 1000. In this reporting period it has been confirmed that a single cosmid clone of several cosmids sampled from the 70 colony sublibrary is strongly positive for growth inhibitory activity. This clone will be referred to as pWM13.

iv) Total RNA was prepared from HeLa cells, WI38 embyro fibroblasts, and several pools of HeLa cells selected by the assay for growth inhibitory sequences. Donor DNAs for these HeLa cell pools were derived by transfection with: E. coli genomic DNA, WI38 genomic DNA, genomic DNA from HeLa cells transfected with WI38 DNA, and WI38 cosmid library DNA. These RNA preparations were examined by Northern analysis using ^{32}P-labeled pWM13 as as probe. Higher levels (at least 10-fold) of a small transcript were detected in WI38 RNA than HeLa RNA; moreover, intermediate levels of this transcript (3-5 fold higher than in HeLa) were observed in each of the HeLa cell pools transfected with DNA containing WI38 sequences. Experiments in which a LINE1 sequence was used as probe confirmed that each of the RNA preparations contained similar quantities of hybridizable material and indicated that the small transcript is probably not related to that repetitive sequence element. Experiments in which the Alu sequence from the clone BLUR8 was used appeared to yield similar results; however, a 7SL RNA cDNA probe (obtained from E. Ullu, Yale University) strongly hybridized to the small transcript. The last result strongly suggests that the small transcript is 7SL RNA. Since 7SL RNA contains Alu-related sequences, we attribute the failure of the BLUR8 probe to hybridize to the presence of regions of non-homology (about 12%) in different Alu clones. To determine the significance of these findings, it will be necessary to demonstrate that the 7SL RNA homologous sequences in pWM13 are responsible for the growth inhibitory activity exhibited by that clone.

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

DNA-mediated transfer of genetic material into mammalian cells is a powerful approach for identifying mechanisms that control cell growth and for elucidating how malfunction of those mechanisms leads to malignant transformation. The project described above is directed towards the systematic improvement of gene transfer technology, as well as towards its simultaneous application to identify genes and/or DNA sequences that regulate mammalian cell growth.

Proposed course:

Further work will focus on refining mammalian vector systems, defining new uses for these systems, and understanding factors which control expression of exogenously introduced genes. Emphasis will be placed on the application of these approaches to the study of mammalian cell growth regulation. Initially, our efforts will concentrate on identification and characterization of DNA sequences that inhibit HeLa cell replication. If this goal can be accomplished, then we will investigate the relationships such sequences may have to differentiation, in vitro senescence, and the control of malignant transformation. Finally, we will attempt to define interrelationships between genes that inhibit and those that stimulate cell growth.

Publications:

Fordis, C.M., Nelson, N., Dean, A., Schechter, A.N., Anagnou, N.P., Nienhuis, A.W., McCormick, M., Padmanabhan, R., and Howard, B.H.: Trans-acting factors are responsible for the lack of β-globin gene expression in K562 cells. In Stamatoyannopoulos, G. and Nienhuis, A.W. (Eds.): Experimental Approaches for the Study of Hemoglobin Switching. New York, A.R. Liss, Inc., 1985, pp. 281-292.

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Protic-Sabljic, M., Whyte, D., Fagan, J., Howard, B.H., Gorman, C.M., Padmanabhan, R., and Kraemer, K.H.: Quantification of expression of linked cloned genes in a simian virus 40-transformed xeroderma pigmentosum line. Mol. Cell. Biol. 5: 1685-1693, 1985.

Reeves, R., Gorman, C.M., Howard, B.: Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells. Nucl. Acids Res. 13: 3599-3615, 1985.

Pozzatti, R., Muschel, R., Williams, J., Padmanabhan, R., Howard, B., Liotta, L., and Khoury, G.: Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. Science 232: 223-227, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08751-06 LMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Regulation of the gal Operon of Escherichia Coli

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

PI:	S. Adhya	Chief, Developmental Genetics Section	LMB, NCI
Other:	A. Majumdar	Visiting Scientist	LMB, NCI
	J. Tokeson	Guest Researcher	LMB, NCI
	M. Polymeropoulos	Visiting Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

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Developmental Genetics Section

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are studying mechanisms of controlling the expression of the gal operon of E. coli. We have demonstrated that the operon is controlled by two promoters, which are modulated by cyclic AMP in opposite ways. We have also previously shown that each of the two gal promoters is negatively regulated by binding of Gal Repressor to two operator elements, one of which (O_E) is located upstream to the promoters and the other (O_I) inside the galE structural gene. O_E and O_I are separated by 114 bp. We have proposed that Gal Repressor acts by affecting promoter structure from distal sites. Repressor bound to O_E and O_I interact by protein-protein contact to generate a DNA loop with altered conformation. The promoter which is part of the loop and thus has undergone conformational change is now inadequate for transcription. Several lines of experimental results are consistent with the model: (1) The precise contact sites in O_E and O_I DNA have been defined by DNase protection, purine methylation protection and phosphate ethylation interference studies. The location of the contact sites suggests that Repressor induces changes in the helical pitch of O_E and O_I DNA, reducing the number of bases per B-DNA helical turn from 10.5 to about 9.0. In a loop structure, any Repressor induced alteration in the two operator segments may be easily transmitted to the promoter region. (2) The total binding energy of simultaneous interaction of Repressor to O_E and O_I is higher than the sum of the intrinsic binding energies to O_E and O_I. The additional energy is easily explained by an interaction between two Repressors occupying O_E and O_I respectively. (3) The formation of a DNA loop requires that the angular orientation of the two Repressor binding sites, O_E and O_I, be favorable for the protein-protein contact. When the angular orientation was sequentially changed by introduction of segments of DNA of different lengths (15 to 37 bp) between O_E and O_I, a recurrence of derepression and repression of the gal promoters with increase of distance between O_E and O_I was observed, as was expected from a DNA loop model.

Project Description

Major Findings:

1. We have previously demonstrated the existence of two operators, O_E and O_I, in the gal operon by genetic analysis and DNA sequencing of the operator constitutive mutants. The binding of Gal Repressor to the operators has been demonstrated in two ways:

   a. By retarded electrophoretic mobilities of operator DNA fragments when bound to Repressor.

   b. By protection of the operator segments from digestion by DNase when bound to Repressor.

2. The two operators are separated by 114 bp and span the promoters. We have previously proposed that Gal Repressor acts by affecting the promoter DNA structure by binding at distal operator sites. Two molecules of Repressor bound to O_E and O_I respectively interact by protein-protein contact to generate a DNA loop structure. This changes the conformation of the promoter to one which is inadequte for transcription.

3. The specific contact points of Repressor in the operators have been identified by protection of contacting purine bases from methylation by Repressor prebinding. The contacting DNA phosphates have also been determined by testing which phosphates interfere with Repressor binding when ethylated by ethylnitroso urea.

4. The structure of the Gal Repressor, based on comparison of amino acid sequences, is analogous to other Repressors of known structure. The location of the phosphates in the operators contacted by Gal Repressor strongly argue that Gal Repressor binding changes the B-DNA helical pitch of the operators. Such changes at O_E and O_I, which are 114 base pairs apart, may be extended to the promoter by the formation of a DNA loop. This change will be sufficient for inactivation of the promoter.

5. We have argued that the formation of a DNA loop, as explained above, requires that the angular orientation of the two Repressor binding sites must be in a favorable configuration for the protein-protein contact. A deviation from that hinders the loop formation and thus causes derepression. To test this prediction, we have sequentially altered the angular orientation of O_E and O_I by introducing segments of DNA of 15 to 37 bp lengths between them. The results show a periodic derepression and repression of the gal promoters with the increase of distance between the two operators. These observations strongly favor a DNA loop formation as the cause of gal repression.

6. We have calculated the interaction energy (ΔG) when Repressor binds individually to O_E and O_I or simultaneously to O_E + O_I by determining the equilibrium constant in each case. The results show that the total

binding energy (ΔG_T) when both $\underline{O_E}$ and $\underline{O_I}$ are simultaneoulsy occupied by Repressor is higher by one KCal over the sum of the intrinsic interaction energies ($\Delta G_E + \Delta G_I$) of each site. The excess energy very likely originates from an interaction between Repressor-$\underline{O_E}$ and Repressor-$\underline{O_I}$ complexes. This is additional evidence in favor of the DNA loop model.

7. In order to define the various functional elements of the Gal Repressor protein, we have undertaken a project which involves isolation and characterization of a large number of Gal Repressor mutants. For this purpose, we have cloned the galR gene in a suitably marked plasmid for easy mutagenesis and subsequent manipulation. Isolation and screening of the mutants are in progress.

Publications:

Adhya, S., Majumdar, A., Polymeropoulos, M. Orosz, L., and Itani, M.: Mechanisms of gal repressor action. In Calendar, R. and Gold, L. (Eds.): Sequence Specificity in Transcription and Translation. New York, Alan R. Liss, Inc., 1985, pp. 245-254.

Adhya, S.: Prokaryotic promoters, activators, and repressors. In Calendar, R. and Gold, L. (Eds.): Sequence Specificity in Transcription and Translation. New York, Alan R. Liss, Inc., 1985, pp. 303-306.

Adhya, S.: The gal operon. In Magasanik, B. and Unbarger, E. (Eds.): Escherichia coli and S. typhimunirum. Washington, D.C., American Society of Microbiology, 1986 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08750-06 LMB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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
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	J. Kim	Biotechnology Fellow	LMB, NCI

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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The cya, crp and rho gene products modulate the expression of a wide variety of bacterial and bacteriophage genes or operons. In order to understand the regulatory processes, we are studying the structure, expression and activity of these genes and gene products. We have previously shown that the protein products of the cya, crp, and rho genes are autogenously regulated. In CRP, cyclic AMP binding to the amino-terminal domain induces an allosteric transition which changes the DNA binding property of the carboxy domains. We have isolated mutants in the crp gene called crp*, which make CRP functional in the absence of cyclic AMP. The mutations responsible for the CRP* phenotype cause substitutions by amino acids with bulkier side chains in the D α-helix of the protein's carboxy domain, near the hinge which connects the carboxy to the amino domain. Apparently, the mutant CRP*s have assumed a conformation that is normally evoked by cAMP binding. We have isolated intragenic suppressors of crp* that restore the need for cAMP. These mutants define precisely the amino acids and thus the parts of CRP which interact specifically to cause the allosteric shift.

We have shown previously by pulse-labeling of RNA and by DNA-RNA hybridization, as well as by operon fusion analysis, that the rho gene appears to be regulated by cAMP and CRP at the levels of transcription and translation. By studying the system in vitro, using coupled and non-coupled transcription/translation systems, we have been unable to show a direct CRP-cAMP effect on rho expression. Since the effect appears indirect, we are currently using a genetic approach to identify the factors involved.

PHS 6040 (Rev. 1/84)

GPO 904-917

Project Description

Objectives:

Gene expression in E. coli and bacteriophage is modulated at the level of transcription initiation, transcription termination, and translation. Cyclic AMP, CRP, and Rho control one or more of these regulatory processes. Rho is involved in transcription termination. cAMP and CRP either stimulate or inhibit transcription initiation. Frequently, the level of these regulatory proteins are critical in gene control. In order to understand the precise molecular basis of these and other regulatory biochemical reactions, we are studying how (a) the synthesis and (b) the activity of these regulatory proteins are regulated.

Methods Employed:

Microbial genetic and biochemical techniques. Also employs both in vivo and in vitro recombinant DNA technology, including dideoxy DNA sequencing method of Sanger.

Major Findings:

1. It is known that CRP undergoes a conformational change when its effector molecule, cAMP, is added. Cyclic AMP binds to the amino terminal portion of the protein. In the cAMP-induced conformation, CRP binds via its carboxy terminal portion to specific sites at the promoters of operons, and causes activation or inhibiton of transcription. Previously, we have isolated mutations in the crp gene that allow CRP to function in the absence of cAMP. These mutations, which we call crp*, continue to be characterized.

    a. Substitution of an amino acid with a bulkier side chain at the hinge region connecting the cAMP-binding N-terminal domain and the DNA-binding C-terminal domain is sufficient to allow CRP* to function without cAMP. This area is not involved in cAMP binding or DNA binding. Depending upon the particular amino acid that is altered in this region, we believe that some mutants have an increased distance between the two domains of each CRP monomer, whereas others have an increased distance between the DNA-contacting F α-helix of the C-terminal domain and the remaining portion of that domain. We think that the CRP*s have, at least in part, assumed the conformational change that is caused by cAMP.

    b. In collaboration with Dr. Joseph Krakow, the CRP* mutant proteins have been studied. Wild type CRP, in the absence of cAMP, is insensitive to a number of proteases such as subtilisin and chymotrypsin. In the presence of cAMP, however, CRP can be cleaved by these proteases, probably because the CRP has undergone a cAMP-induced conformational change. The CRP* mutant proteins, without cAMP, are

sensitive to these proteases, suggesting that these mutant proteins have assumed something similar to the cAMP-induced wild-type conformation.

c. Starting with two separate crp* mutants, we isolated intragenic suppressors of the crp* mutation. The crp* mutation allows, for example, lac expression in a strain which lacks cAMP. In contrast, the intragenic suppressor mutant, although the crp* mutation is present, is phenotypically like wild-type CRP would be in a cya^- strain--it is Lac^-. The crp*-suppressor mutations were mapped by sequencing DNA of the mutant gene. For crp* mutation at amino acid 141, one *-suppressor mutation mapped at amino acid 127. Since amino acid 127 in the wild-type protein, from x-ray crystallography data, is thought to be involved in stabilization of the two subunits of a CRP dimer, we believe the crp* 141 mutation is suppressed by mutation at position 127 because the subunits cannot align properly. The net result is that the protein again requires cAMP, which itself plays a role in subunit alignment. For another crp* mutation (at position 144), the suppressor mutation actually consisted of two base changes, resulting in changes at amino acids 169 and 171. We do not know yet whether both mutations are necessary to suppress the 144 mutation. Amino acid 171 in the wild-type protein in x-ray crystals forms a hydrogen bond with an amino acid in the cAMP-binding amino terminal domain of the protein. We think that the crp*-144 mutation is suppressed by the mutation at 171, although the crp*-mutation would cause the proper conformation in the absence of cAMP, because the domains within the subunit would not be aligned properly. These crp* and crp^{*-sup} mutations have allowed us to develop a model for how cAMP causes the allosteric change within CRP.

d. To try to identify which particular amino acids are involved in the cAMP-induced allosteric change of CRP, we have begun to isolate a number of crp mutations which we call $Allo^-$. These mutants have lost the ability to undergo the allosteric change. These crp mutations produce CRP to which cAMP can bind, but which cannot bind to DNA. We mutagenized the wild-type crp^+ gene on a plasmid and then transformed a crpΔ host. We selected those which remained Crp^- after transformation and screened crude extracts from these crp^--plasmid containing strains for retention of cAMP-binding. Those that could bind cAMP like wild-type are currently being sequenced. Since DNA contact points within CRP are well documented, it is likely that any mutations we obtain that are not of the DNA contact type are likely to be those which have mutations affecting the allosteric shift.

2. We had isolated previously a number of potential $cGMP^-$ mutants. These mutants were isolated starting with a crp* cyaΔ strain under an assumption that the CRP* was using cGMP as an effector. We selected those which lost the ability to express the lactose and sorbitol operons but could express them if exogenous cGMP were added. They are potential guanylate cyclase or guanylate cyclase regulatory mutants. We are currently mapping these mutations using Tn10 transposons. Once we have

established the nature of these mutants, we may be able to define the role of cGMP in E. coli. Cyclic GMP is an important regulator in animal cells. Although it is present in E. coli, its role is not known. Interestingly, several of our potential cGMP$^-$ mutants are temperature-sensitive for growth, suggesting that the mutation lies in an essential gene.

3. We have shown previously that strains having mutations in the cya or crp genes make more Rho protein than do wild-type cells, but there is less rho mRNA in the mutant cells. This observation is consistent with a model of rho regulation involving CRP/cAMP dependent transcriptional and translational control of rho

To elucidate the mechanism by which CRP/cAMP controls rho, active S-30 systems were prepared from wild-type, crp, and rho$^-$ strains. We studied rho gene expression directed by a rho DNA template in the S-30 extracts when transcription and translation were both coupled and uncoupled. Under all condtions tested, direct addition of CRP/cAMP to the S-30 extracts had no effect on rho expression. In contrast, addition of Rho protein to this system had a negative effect on rho expression. This Rho autoregulation had been shown previously in vivo.

From this result we conclude that the effect of CRP/cAMP on rho could be indirect, involving other factor(s) under CRP/cAMP control. We are currently constructing a set of plasmids which contain either the rho promoter or internal regions of the rho gene, fused to lacZ. These constructions will be used in two types of studies. First, crp$^-$ or cya$^-$ cells will be transformed with these plasmids in order to identify regions of the rho gene involved in CRP/cAMP mediated transcriptional and translational regulation. Next, the rho-lacZ plasmids will be used in genetic studies to identify the presumed CRP/cAMP dependent factor(s) which regulate rho.

Significance to Cancer Research and the Program of the Institute:

National Cancer Plan Objective 3, Approach 1

In cancer cells the expression of some genes are permanently turned on, i.e., expressed contitutively and some genes are permanently turned off, i.e., never expressed. Our studies are aimed at understanding the molecular basis of how genes are turned on and off and how genetic regulatory elements interact with each other. This understanding might help to prevent conversion of normal cells to those capable of forming cancers.

Proposed Course of Research:

(1) To determine how cAMP regulates the activity of CRP.

(2) To determine the regulation of and the role of cGMP in E. coli.

(3) To determine how the rho gene of E. coli is regulated.

Publications:

Garges, S. and Adhya, S.: Site of allosteric shift in the structure of the cyclic AMP receptor protein. Cell 41: 745-751, 1985.

Gulletta, E., Gavriella, S., and Adhya, S.: Cloning and expression of the Escherichia coli rho gene in a plasmid vector. Microbiologia 8: 303-312, 1985.

Garges, S. and Adhya, S.: How the cyclic AMP receptor protein of Escherichia coli works. In Thompson, E.B. and Papaconstantinou, J. (Eds): DNA: Protein Interactions and Gene Regulation. Austin, Texas, University of Texas Press, 1986, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08710-10 LMB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

CHECK APPROPRIATE BOX(ES)

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

B

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

The molecular mechanisms involved in DNA replication are being studied biochemically. The system used is the in vitro replication of E. coli phage λ plasmid DNA. This reaction requires two phage initiation proteins, O and P gene products, many host proteins including DnaB, DnaG primase, pol III holoenzyme, DnaJ, DnaK, DNA gyrase, DNA ligase and Ssb, RNA transcription in the region of the origin of replication, and a specific site, ori λ, on the DNA. I have been isolating the proteins required for this reaction with the long range goal of reconstituting the λ replication pathway with all purified proteins. Currently, I am purifying DnaJ and DnaK proteins by in vitro complementation assays and characterizing them biochemically. DnaK has weak ATPase activity, DnaJ is a DNA binding protein and both form protein complexes with λ P protein. I am participating in a collaborative electron microscopic study of nucleoprotein structures formed by λ O, λ P, DnaB, Ssb, DnaJ and DnaK proteins at the origin of λ replication. I am also characterizing the DNA binding domain of the λ O protein.

GPO 914-918

Project Description

Objectives:

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

Methods Employed:

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

Major Findings:

I have been studying the E. coli and λ proteins involved in the specific initiation of replication at the λ origin. In collaboration with Mark Dodson and Hatch Echols, we have observed by means of electron microscopy, specialized nucleoprotein structures at the λ origin of replication. Previous work of others had shown that λ O protein binds to four 19 base pair DNA binding sites in the λ origin region and that DnaB and λ P proteins generate a larger complex with λ O in an ATP-requiring reaction. Our recent studies have shown that the addition of DnaJ, DnaK and Ssb proteins results in an origin specific DNA unwinding reaction. This reaction requires ATP and is probably catalyzed by the helicase activity of DnaB protein. DNA gyrase increases the extent of the unwinding. The unwinding reaction is unidirectional, proceeding rightward from the λ O binding sites in the origin. The factors required for bidirectional replication remain unknown. Replication, transcription or another cellular protein might be required to generate leftward unwinding. The minimal λ DNA sequence that is a competent substrate for unwinding consists of the two right most O binding sites and most of the adjacent A + T rich region to the right of the O binding sites. These results parallel my in vitro DNA replication experiments that show the same minimal DNA sequence to be sufficient for initiation of λ replication. Thus, it appears that the λ O protein localizes and initiates a six protein sequencial reaction responsible for but preceeding the initiation of replication. Presumably the first step is the binding of O protein to the origin through its amino-terminal DNA binding domain. In studies with Mark Dodson, we are looking at complexes of the amino-terminal O protein fragment with λ origin DNA. Very likely, DnaB protein is brought to the λ origin through protein complexes formed between it and P and between P and the carboxy-terminus of O. I have demonstrated these protein-protein interactions biochemically. The role of DnaJ and K proteins is still vague. Since they both interact with DnaB, it is likely they are involved in transferring DnaB from P protein to DNA at the origin. Once on the DNA, DnaB serves as a mobile promoter for primer synthesis by DnaG. Primer elongation is then catalyzed by DNA pol III holoenzyme. The role of RNA transcription in initiation is still not understood.

I have also been characterizing biochemically DnaJ and K proteins. These two proteins are induced during heat shock and are required for λ replication but their function in E. coli is not known. I have purified DnaJ and K using in vitro complementation assays in which crude extracts prepared from either dnaJ or dnaK temperature sensitive cells, when supplemented with O and P proteins, catalyze λ replication upon the addition of DnaJ or K protein respectively. While I do not yet know their roles in replication, it is clear from the electron microscopic work described above, that they are involved in preinitiation reactions. My experiments have shown both DnaJ and DnaK form protein complexes with λ P protein. Indirect experiments suggest that K releases DnaB from a tight complex with P.

I have finished the work on the biochemical characterization of the DNA binding domain of the amino-terminal portion of λ O protein. However, I am still collaborating with Douglas Olendorf in attempting to crystallize the amino terminal O protein fragment. Because he has moved to a new laboratory at Dupont, progress has been slow. We are eventually hoping to learn the structure of O in the presence and absence of DNA.

Significance to Cancer Research and the Progam of the Institute:

The central process of heredity and cell growth is the replication of the genetic material. Studies of this process in E. coli have been made possible by the availability of mutants defective in DNA synthesis and large amounts of bacteria necessary for biochemical studies. The understanding that this work is generating about the biochemical mechanisms of DNA replication in E. coli has, and will continue to, shed light on the nature of the same reactions in animal cells.

Proposed Course of Research:

I will continue studying biochemical mechanisms involved in DNA replication using purified proteins and defined DNA templates. I will use the in vitro λ replication model system to study the events occurring at initiation including the protein-DNA interactions, the protein-protein interactions and the ATP requirements.

Publications:

Wickner, S., and Zahn, K.: Characterization of the DNA binding domain of bacteriophage λ O protein. J. Biol. Chem. 1986 (in press).

Dodson, M., Echols, H., Wickner, S., Alfano, C., Mensa-Wilmot, K., Gomes, B., Lebowitz, J., Roberts, J., and McMacken, R.: Specialized nucleoprotein structures at the origin of replication of bacteriophage λ : Localized unwinding of duplex DNA by a six protein reaction. Proc. Natl. Acad. Sci. USA 1986 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08010-13 LMB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Morphologic Mechanisms of Organelle Function and Transformation in Cultured Cells

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

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

COOPERATING UNITS *(if any)*

Laboratory of Cell Biology, DCBD, NCI
Metabolism Branch, DCBD, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

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 transformation-related areas: 1) We have employed light microscopic immunocytochemistry to evaluate monoclonal antibodies for suitability as immunotoxin reagents for cancer chemotherapy. We adapted methods for light microscopic immunocytochemistry in whole tissues, using frozen human tissues, cryostat sections and peroxidase labeling methods. This standardized method has been used to evaluate HB-21 (a monoclonal antibody to the human transferrin receptor), anti-TAC (a monoclonal antibody to the human IL-2 receptor), and other monoclonal antibodies generated against human ovarian cancer cell lines. 2) We have studied the phenomenon of multidrug-resistance (MDR) in cultured cells and morphologically examined the accumulation of the chemotherapeutic drug daunomycin. Using this drug as a fluorescence marker of drug accumulation in cells in culture, photometric microscopy studies of nuclei in individual cells showed that MDR cells have an accelerated efflux mechanism that is energy-dependent. This rapid morphologic assay has made it possible to evaluate multiple drugs for their effects on this efflux activity, and has shown major inhibition of daunomycin efflux by verapamil, quinidine, and vinblastine. 3) We have used immunocytochemical methods to localize some major proteins in transformed cultured cells. We have localized "p55", a major protein in cells that shows thyroid hormone binding activity, and "p86", a cytosolic heat-shock protein present in large amounts in many cells and identified as a tumor-specific transplatation antigen in animal systems. In other studies, preliminary experiments using single cell microinjection methods have demonstrated a specific inhibition of epidermal growth factor receptor synthesis following injection of anti-sense RNA for the EGF receptor gene.

PHS 6040 (Rev. 1/84)

GPO 904-917

Other Professional Personnel:

I. H. Pastan	Chief, Laboratory of Molecular Biology	LMB, NCI
M. M. Gottesman	Chief, MCG Section	LMB, NCI
D. J. FitzGerald	Senior Staff Fellow	LMB, NCI
L. Beguinot	Visiting Fellow	LMB, NCI
M. Cornwell	Guest Researcher	LMB, NCI
S.-y. Cheng	Research Chemist	LMB, NCI
N. D. Richert	Microbiologist	LMB, NCI
S. J. Ullrich	Guest Worker	LCBGY, NCI
E. Appella	Chief, Chemistry Section	LCBGY, NCI
T. W. Waldmann	Chief, Metabolism Branch	MET, NCI

Project Description

Objectives:

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

Methods Employed:

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

Major Findings:

We have studied the distribution of cell surface antigens in culture on certain tumor cell types and have adapted methods for the light microscopic detection of antigens in tissues. This latter approach uses immunoperoxidase labeling and cryostat sections to evaluate multiple monoclonal antibodies for their usefullness as immunotherapeutic reagents. We have evaluated the tissue distribution of the human transferrin receptor (using monoclonal HB-21), the human IL-2 receptor (using anti-TAC monoclonal antibody), and antigens that react with a series of ovarian tumor-specific monoclonal antibodies (OVB-1, etc.). These studies have shown that the transferrin receptor is ubiquitously distributed in various tissues and is particularly concentrated in hepatocytes, epithelia of the GI and urinary tracts, skin, and the endothelium of brain capillaries. These results suggest that immunotoxins made using monoclonal antibodies to the human transferrin receptor would have more potential

toxic side effects than previously thought. The IL-2 receptor was found to be distributed in very few normal tissues, but a major population of cells that react with anti-TAC antibody were found in a number of tissues as interstitial cells (perhaps macrophages), particularly prominent in thyroid. OVB-1 and other OVB antibodies were found to react with various tissue elements; a major location of OVB-1 was found on the surface of polymorphonuclear leukocytes in many tissues. Another protein localized in tissues was nematin, a common intermediate filament protein of unknown function in cultured cells. Nematin was found in an intermediate layer of the epidermis, and in unique cells in the cerebral cortex and cerebellum.

Multidrug-resistance (MDR) of human tumor cells was examined using cultured MDR derivatives of KB cells. We employed fluorescence microscopy to detect the accumulation of daunomycin, an inherently fluorescent anti-tumor drug. Using microscope photometry we demonstrated that a major cause of the lack of accumulation of chemotherapeutic drugs in MDR cells was the existence of a highly efficient energy-dependent efflux pump at the plasma membrane of MDR cells. Using this as a rapid assay of the effects of other agents on this efflux activity, we found that verapamil, quinidine, and vinblastine were all effective in inhibiting daunomycin efflux. This result was confirmed by the restoration of sensitivity to chemotherapeutic drugs in cell growth experiments when some of these other agents were including in the culture medium of MDR cells. The method of examining fluorescent daunomycin accumulation in living cells will allow a simple and rapid screening of other drugs for their effect on MDR cell efflux activity. Such agents of low toxicity would be potentially useful as adjunctive agents in human cancer chemotherapy. A protein implicated in this efflux activity of MDR cells, gp170, has been localized in preliminary experiments using immunocytochemistry to the plasma membrane and other intracellular membranous structures.

We have localized some major cell proteins of special interest in intracellular sites using light and electron microscopic immunocytochemistry. One of these, p55, is a protein that shows significant specific binding activity for thyroid hormones. This protein was localized using monoclonal antibodies and found to be present exclusively in the endoplasmic reticulum and nuclear envelope. The significance of this distribution in thyroid action is still under investigation. Another protein localized was p86, a major tumor transplantation antigen thought to reside on the surface of some cells. Localization of this protein revealed it to be mainly localized in the cytosol, and further studies showed it to be a major heat-shock protein ubiquitously distributed in many cells. A small amount of this protein was found on the surface of cells, but this distribution appears to be the result of death of adjacent cells in the culture which release large amounts of this "sticky" cytosol protein into their surroundings. None of this protein was found in the intracellular membrane-limited organelles involved in the synthesis and processing of proteins destined for the cell surface.

Microinjection studies have been used to evaluate the effects of injecting anti-sense RNA for the human EGF receptor into the cytoplasm of living cells. Preliminary results have shown that the sythesis of new EGF receptor can be

specifically blocked by the presence of this injected anti-sense RNA. Further studies are directed at examining the effect of this inhibition of receptor synthesis on growth and other cell properties.

Significance to Cancer Research and the Program of the Institute:
National Cancer Plan Objective 6, Approach 3.
Understanding basic cellular mechanisms of organelle function and composition is central to the full understanding of the functions of tumor cells, and how they differ from normal cells. In addition, the understanding of the cellular mechanisms of response to immunotherapeutic and chemotherapeutic agents is essential to the proper design and application of these new modalities of cancer therapy.

Proposed Course:

We will continue to study the surface composition of tumor cells and monoclonal antibodies directed to them in an attempt to select specific reagents that will be of use in immunotoxin therapy of human cancer. In addition, we will continue to evaluate chemical agents for their effects on the MDR efflux pump activity with the aim of finding drugs that would have low overall toxicity to normal tissues, yet would inhibit the resistance of MDR cells to chemotherapeutic drugs. We will examine in more detail the location and expression of gp170 in MDR cells. We will also attempt to design experiments to evaluate the importance of the EGF receptor in growth control in human epithelial cells using anti-sense RNA injection to inhibit the production of new receptor.

Publications:

Pirker, R., FitzGerald, D.J.P., Hamilton, T.C., Ozols, R.F., Laird, W., Frankel, A.E., Willingham, M.C., and Pastan, I.: Characterization of immunotoxins active against ovarian cancer cell lines. J. Clin. Invest. 76: 1261-1267, 1985.

Goldenthal, K.L., Pastan, I., and Willingham, M.C.: Serial section analysis of clathrin-coated pit morphology in rat liver sinusoidal endothelial cells. Exp. Cell Res. 161: 342-352, 1985.

Pirker, R., FitzGerald, D.J.P., Hamilton, T.C., Ozols, R.F., Willingham, M.C., and Pastan, I.: Anti-transferrin receptor linked to Pseudomonas exotoxin as a model immunotoxin in human ovarian carcinoma cell lines. Cancer Res. 45: 751-757, 1985.

Chen, J.W., Murphy, T.L., Willingham, M.C., Pastan, I., and August, J.T.: Identification of two lysosomal membrane glycoproteins J. Cell Biol. 101: 85-95, 1985.

Willingham, M.C. and Pastan, I. Immunofluorescence techniques. In Pastan, I. and Willingham. M.C. (Eds.): An Atlas of Immunofluorescence in Cultured Cells. Orlando, Florida, Academic Press, 1985, pp. 1-13.

Goldenthal, K.L., Hedman, K., Chen, J.W., August, J.T., and Willingham, M.C.: Post-fixation detergent treatment for immunofluorescence suppresses localization of some integral membrane proteins. J. Histochem. Cytochem. 33: 813-820, 1985.

Willingham, M.C. and Pastan, I.: Receptosomes, endosomes, CURL: different terms for the same organelle system. Trends Biochem. Sci. 10: 190-191, 1985.

Hanover, J.A., Beguinot, L., Willingham, M.C. and Pastan, I.H.: Transit of receptors for epidermal growth factor and transferrin through clathrin-coated pits: analysis of the kinetics of receptor entry. J. Biol. Chem. 260: 15938-15945, 1985.

Pastan, I., Hanover, J., and Willingham, M.C.: The cellular entry of EGF and transferrin: a problem in intracellular sorting. In Ginsburg, R.A. and Levine, R.L. (Eds.): Current Topics in Cellular Regulation, Vol. 26. Orlando, Florida, Academic Press, 1985, pp. 17-25.

Hedman, K., Pastan, I., and Willingham, M.C.: The organelles of the trans domain of the cell: ultrastructural localization of sialoglycoconjugates using Limax flavus agglutinin. J. Histochem. Cytochem. 1986 (in press).

Beguinot, L., Werth, D., Ito, S., Richert, N., Willingham, M.C., and Pastan, I.: Functional studies on the EGF receptor with an antibody that recognizes the intracellular portion of the receptor. J. Biol. Chem. 261: 1801-1807, 1986.

Cheng, S.-y., Hasumura, S., Willingham, M.C., and Pastan, I.: Purification and characterization of a membrane-associated 3,3',5-triiodo-L-thyronine binding protein from a human carcinoma cell line. Proc. Natl. Acad. Sci. U.S.A. 83: 947-951, 1986.

Ullrich, S.J., Robinson, E.A., Law, L.W., Willingham, M., and Appella, E.: A mouse tumor-specific transplantation antigen is a heat-shock-related protein. Proc. Natl. Acad. Sci. U.S.A. 1986 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08752-06 LMB

PERIOD COVERED
Octoboer 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanism of the Transport of Thyroid Hormones into Animal Cells

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

PI:	S.-y. Cheng	Research Chemist	LMB, NCI
Others:	I. Pastan	Chief, LMB	LMB, NCI
	Clifford Parkison	Chemist	LMB, NCI
	S. Kitagawa	Visiting Fellow	LMB, NCI
	T. Obata	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:
4.1	4.1	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.)*

A membrane-associated thyroid hormone binding protein (p55) has previously been purified to homogeneity from cultured A431 human epidermal carcinoma cells. Partial sequences from the N-terminal and a cyanogen bromide fragment of p55 were determined. One polyclonal and four monoclonal antibodies against p55 were used to screen the λgt11 cDNA libraries prepared from human A431 and KB cells; 22 and 13 positive plaques were obtained, respectively. The size of the insert ranges from 0.5-1.8 Kb. Using 1.8 Kb insert as a probe to screen a human cDNA library from fibroblast cells, a clone with a 2.2 Kb insert was obtained. This size is similar to that of mRNA of p55 determined by Northern blot analysis. The 5' sequence of 2.2 Kb fragment was determined which contains the initiation codon and the N-terminal sequence of p55. The apparent full-length cDNA clone for the thyroid hormone binding protein is being characterized.

Analysis of the cellular extracts of A431 cells indicated the presence of another binding protein for 3, 3', 5-triiodo-L-thyronine (T_3). In contrast to p55, this binding component has an apparent MW of 58K and a PI of 7.1. By a combination of Sepahdex G-200, OAE, SP-Sephadex and hydroxyapatite column chromatography, p58 was purified to near homogeneity. Antibodies to p58 are being prepared and its cellular functions will be evaluated.

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

Project Description

Objectives:

To elucidate the mechansim of the entry of thyroid hormones into animal cells at molecular level. To study the mechanism of the translocation of thyroid hormone from cytoplasm into nucleus where the initiation of biological actions occurs. To understand the roles of the cellular thyroid hormone binding proteins on cellular growth promoting effects by thyroid hormones.

Methods Employed:

Use DNA recombinant techniques to clone, identify and characterize the gene for p55. Use dideoxy sequencing techniques to determine the nucleotide sequence of the gene for p55. Use immunoprecipitation and metabolic labeling to identify the translation products from the gene of p55.

Use radiolabeled T_3 to quantify and characterize the binding of thyroid hormone to the detergent solubilized binding proteins. Photoaffinity labeling was used to covalently link the hormone to the binding protein. Chromatographic techniques were used to separate, characterize, and purify the thyroid hormone binding protein. Elisa and radioimmuno assays were used to screen antibodies against the thyroid hormone binding proteins.

Major Findings:

Using recombinant DNA techniques, an apparent full-length cDNA clone for p55 was found after screening three human cDNA libraries. This clone has a 2.2 Kb insert. Sequencing analysis indicated the presence of initiation codon (ATG) followed by a coding region of 45 bp for hydrophobic amino acids. Thereafter, a coding region for the N-terminal sequence of p55 was detected. The sequencing for the gene of p55 is ~50% completed.

During the purification of p55, another T_3 binding protein was found in the A431 cellular extracts. Using $[^{125}I]T_3$, binding of thyroid hormone to this protien was evaluated. Binding of T_3 to this protein is saturable. T_3 binds to this protein with a Kd of 2.5 nM and Bmax of 1.34 pmoles/15 µg protein. A similar Kd and Bmax for the binding of L-thyroxine was also observed. The The pH optimum for T_3 binding is 6.6. Using the underivatized inner-ring ^{125}I-labeled L-thyroxine as a photoaffinity-labeling reagent, this protein was found to have an apparent molecular weight of 58,000. Isoelectric focusing indicated p58 has a PI of 7.1. Treatment of p58 with N-glycanase, endoglycanase, and mixture of neuraminidase and glycosidase failed to indicate that p58 is a glycoprotein. By a combination of Sepahdex-G-200, OAE, SP-Sephadex and hydroxyapatite column chromatography, p58 was purified to near homogeneity.

Significance:

National Cancer Plan Objective 5, Approach 5.

Proposed Course:

We will continue to study the molecular mechanism of the transport of thyroid hormones into animal cells. The role(s) of p55 and p58 thyroid hormone binding proteins on the T_3 transport and T_3 growth promoting activities will be evaluated. The purified p58 will be sequenced. Oligonucleotides with the sequences corresponding to the N-terminal and other sequences of p58 will be synthesized and used as probes to clone the gene of p58. Polyclonal and monoclonal antibodies to p58 will be prepared. These antibodies could be used not only for intracellular localization of p58, but also for screening of cDNA libraries. The cDNA clones for p55 and p58 can be used to prepare anti-sense RNA and/or to transfect the target cells for studying the function and regulation of the thyroid hormone binding proteins.

Publications:

Lai, C.S., Korytowski, W., Niu, C.H., and Cheng, S.-y.: Transverse motion of spinlabeled 3,3',5-triiodo-L-thyronine in phospholipid bilayers. Biochem. Biophys. Res. Commun. 131: 408-412, 1985.

Hasumura, S., Kitagawa, S., Pastan, I., and Cheng, S.-y.: Solubilization and characterization of a membrane 3,3',5'triiodo-L-thyronine binding protein from rat pituitary tumor GH_3 cells. Biochem. Biophys. Res. Commun. 133: 837-843, 1985.

Stevens, R.L., Hasumura, S., Parsons, W.G., and Cheng, S.-y.: The identification of a plasma membrane 3,3',5-triiodo-L-thyroinie binding protein in the culture Swarm rat chondrosarcoma chondrocyte and the lack of its up regulation by insulin in vitro. Endocrinology 118: 573-582, 1986.

Cheng, S.-y., Hasumura, S., Willingham, M.C., and Pastan, I.: Purification and characterization of a membrane associated 3,3',5'triiodo-L-thyronine binding protein from a human carcinoma cell line. Proc. Natl. Acad. Sci. USA 83: 947-951, 1986.

SUMMARY

Annual Report of the Laboratory of Biochemistry, National Cancer Institute

October 1, 1985 to September 30, 1986

I. INTRODUCTION

The investigations carried out in this Laboratory utilize the techniques of genetics, biochemistry and molecular biology to investigate fundamental questions about the biology of organisms ranging from bacteria through yeast, invertebrates and mammals including humans. Such studies illuminate not only normal biological processes but also the aberrant events associated with disease and in particular, cancer. Because new techniques frequently permit investigators to approach new problems or old problems in an innovative way, emphasis is given to the development of new methodologies. Overall, the activities within the Laboratory are diverse in their specifics, but unified by overlapping common interests and a sharing of knowledge and skills.

During this year, the Chief of the Laboratory, upon the advice of the Section Chiefs, proposed Cary Queen, Ph.D. for conversion from a Senior Staff Fellow to a permanent member of the Laboratory staff. We were very pleased when the Division and then the Scientific Directors approved of this conversion. This appointment brings to the Laboratory additional strength in the area of the regulation of gene expression, particularly as it relates to the immune system and the maturation of lymphocytes.

Dr. Roberto Di Lauro of the IInd Medical School, Naples, Italy was a visitor in the Laboratory during this year. His work on the regulation of expression of thyroglobulin gene in thyroid cells was significantly extended during his stay. The work related well to ongoing projects on tissue-specific gene expression and the Laboratory staff benefited from Dr. DiLauro's knowledge and insights, and from his introduction of highly useful technical innovations.

The staff of the Laboratory has been extremely cooperative in the ongoing effort to meet the budgetary cut-backs imposed this year. The procedures introduced to curtail operating costs were effective and thus far have not significantly altered the productivity of the working staff; additional cut-backs are likely to have a substantial effect. Nevertheless, overall productivity is hampered by the substantial decrease in the size of the staff that has resulted from restrictions on hiring imposed over the last few years. The central issue here is the same as that raised in last year's Annual Report: the decreased availability of positions for Staff Fellows. To try to ameliorate this problem we applied for and received from the Josiah Macy Jr. Foundation, funds for two post-doctoral fellows. This fellowship program is specifically designed to foster careers in research for female scientists, an area of particular concern to the Macy Foundation. While these two fellowships have allowed us to recruit two fellows for next year, the continuing productivity of the Laboratory must be supported by adequate funding of post-doctoral fellows by intramural NIH resources.

II. BIOSYNTHESIS SECTION (Dr. E.L. Kuff, Chief)

The Section has four principal investigators, each of whom is carrying out an independent research program. In addition, there is collaboration between several members of the section.

A. Dr. Beverly Peterkofsky and coworkers: Experiments summarized in last year's Annual Report led these investigators to propose a model for the pathophysiology of scurvy in which the observed defects in collagen and proteoglycan synthesis are caused indirectly by ascorbate deficiency. They suggested that the decreases were caused by changes in humoral factors during scurvy-induced fasting, rather than through the role of ascorbate in the biosynthetic pathway of either of these components; it was previously assumed that defective connective tissue metabolism in this disease was due to defective proline hydroxylation and procollagen secretion. The results of current studies provide additional support for the new model: changes in hormones and growth factors found in the serum of scorbutic fasting guinea pigs are similar to the changes observed in fasted animals and humans with no ascorbate deficiency. The exposure of normal cartilage chondrocytes to such scorbutic serum, with supplementation by ascorbate, resulted in decreased synthesis of extracellular matrix components. The collagen synthesized had normal levels of hydroxyproline. Thus, the growth promoting activity of vitamin C in vivo appears to be mediated initially through reactions other than those directly involved in connective tissue metabolism. These findings represent a major revision in long-standing concepts of the pathogenesis of scurvy.

In a second study, these workers found that the collagen phenotype of a 4-nitroquinoline-1-oxide transformed line of Syrian hamster embryo (SHE) fibroblasts, NQT-SHE, was markedly altered from that of normal SHE cells, which synthesized mainly type I procollagen, $(pro\alpha 1)_2 pro\alpha_2$. Total collagen synthesis in the transformant was reduced to about 30% of the control level primarily because synthesis of one of the two subunits of type I procollagen, $pro\alpha 1(I)$, was completely suppressed. The major collagenous products synthesized consisted of two polypeptides, designated as N-33 and N-50, which could be completely separated by precipitation with ammonium sulfate at 33% and 50% saturation, respectively. Results of peptide mapping and other experiments indicate the N-collagens are altered $pro\alpha 2(I)$ chains and that they differ from each other.

B. Dr. Samuel Wilson and coworkers: As part of a long-standing program concerned with the mechanism of DNA replication in mammalian cells, these investigators continued studies of cDNAs for the replication proteins alpha-polymerase and beta-polymerase, and for a single-stranded nucleic acid binding protein (helix destabilizing protein). The cDNA to rat brain alpha polymerase described last year was sequenced and found to contain only a short open reading frame, presumably corresponding to the C-terminal end of the alpha polymerase catalytic polypeptide. Antiserum raised against a synthetic oligopeptide with this sequence reacted with purified alpha polymerase and immunoprecipitated alpha polymerase from solutions of crude enzyme. cDNAs from a poly A^+ RNA library of human cells were selected using the antibody to alpha polymerase. cDNAs are being used as probes to study gene expression and Southern blot analysis of genomic DNA is being used to identify polymorphisms and determine chromosomal localizations.

The nucleotide sequence of a cDNA to rat beta polymerase was also determined. Properties of the sequence 5' of the first ATG in the cDNA suggested that this ATG is the initiation codon for synthesis of the 318 amino acid protein encoded by the open reading frame of the mRNA. This cDNA was used to probe a cDNA library of poly A^+ RNA from a human cell line and an analogous human cDNA was isolated and sequenced. The human and rat cDNAs are 90% homologous and the deduced 318 amino acid proteins are similar (95% matched residues).

The full-length cDNA for the binding protein was subcloned in an expression vector and the protein was over-produced in E. coli and isolated in mg quantities. The amino acid sequence of this protein is identical with the sequence of the protein deduced from the open reading frame of the cDNA and with the sequence of the A1 protein purified from mammalian hnRNP particles (see below). Protein-nucleic acid binding studies revealed that the C-terminal domain of the protein is critical for tight binding.

C. Drs. Kuff and Lueders, and coworkers: These investigators continued studies of the intracisternal A-particle (IAP) genes, a genetically distinctive type of retrovirus-like element or retrotransposon that is extensively reiterated in the genomes of mouse and other rodent species. (The family of mouse IAP elements, about 1000 per haploid genome, includes a number of structural variants with deletions and/or insertions at characteristic positions.) Last year's Annual Report described a collaborative study with Drs. K. Moore and K. Ishizaka in which a cDNA clone encoding an IgE-binding factor (a form of T-cell-secreted IgE-regulatory protein) was identified as a member of the IAP gene family. In the past year, 5 additional cDNA clones that code for IgE-binding factors were characterized. Each one proved to be a different structural variant of the full-length IAP proviral element. Antisera against the IAP core protein p73 reacted with the IgE-binding factors, and isolated p73 itself binds to IgE and potentiates IgE production in immunized B-lymphocytes. Several cDNA clones were isolated from normal mouse thymus by immuno-selection for the production of proteins cross-reactive with p73 and are being compared with the IgE-binding factor clones by sequence analysis. Nucleotide sequencing of a full-size 7.3 Kb mouse IAP genomic element has been completed. The mouse IAP sequence has strong homology with that of the Syrian hamster (reported by other investigators) throughout the 3' portion of the gag coding region (p27, p12 and protease) and the entire pol region (reverse transcriptase and endonuclease). There is no homology between the mouse and hamster elements in the 5' portion of gag, including the region that codes for the IgE binding factor in mouse cDNA clones. The data suggest that in the mouse, a cellular form of an IgE regulatory protein gene may have been incorporated into the IAP gag coding region and become part of the 73,000 Da core protein of this defective retrovirus.

In other IAP related studies, work was completed showing that in vitro DNA methylations at a cluster of three HhaI sites in the 5' region of a cloned IAP LTR completely abolished the capacity of the LTR to promote CAT gene expression in an appropriately constructed recombinant plasmid while methylation at a single HpaII site very near the 3' end of the LTR reduced activity by 75%. These observations define a system for elucidating the mechanism by which methylation inhibits IAP and other retroviral expression.

An earlier Annual Report described a nuclear immunostaining reaction obtained with one of the rabbit antisera prepared against the IAP core protein p73. The nuclear components responsible for this reaction have now been identified by Western blot analysis as the hnRNP proteins A1 and A2. Antibodies affinity-purified by binding to the cloned A1 protein produced in E. coli (see Dr. Wilson's summary above) reacted with the IAP protein. The shared epitope may be related to the fact that both the hnRNP and IAP proteins have binding capacity for single-stranded RNA. Under certain growth conditions, A1 and A2 are markedly depleted in nuclei of cultured mouse fibroblasts, suggesting that these proteins may be produced in rate-limiting amounts in rapidly growing cells.

D. Dr. Lueders: Studies dealing with the structure and function of the type II IAP elements were continued. These elements can be distinguished from the more abundant type I IAP elements by a specific 290 bp insertion designated AIIins. Sequencing of several type II IAP elements revealed that part of the region coding for retroviral endonuclease is duplicated and has made it possible to isolate and clone sequences containing only AIIins for use as a specific probe for these elements. Using this probe with Southern blots of mouse genomic DNA, Dr. Lueders has shown that: (1) association with other repetitive sequences appears to be a general property of many type II IAP elements; (2) amplification of type IIB elements is 6-fold in MOPC-315 myeloma and 2-fold in MOPC 104E myeloma relative to embryo DNA; (3) partial demethylation of type II elements in myeloma cells correlates with the known transcriptional activity of the element subclasses. Other results suggest that association with L1 repeats is a feature of transcriptionally silent type II IAP elements, and may reflect their location in a particular chromosomal environment. Multiple copies of AIIins were inserted into vectors containing RNA promoter sequences to produce single strand RNA probes. Using these to probe Northern blots, antisense mRNA was detected in a variety of normal tissues which do not express type II IAP sense message but do express type I IAP mRNA. The antisense transcripts in mouse cells had discrete sizes of 4.6 and 2 Kb; Syrian hamster cells contained a 5.2 Kb antisense RNA in addition to these.

III. CELLULAR REGULATION SECTION (Dr. O. Wesley McBride, Chief)

The work of the Cellular Regulation Section is carried out by two independent groups.

A. Dr. O. Wesley McBride and coworkers: A large group of interspecific somatic cell hybrids segregating human chromosomes was previously isolated and characterized by isoenzyme and cytogenetic analyses. Southern analysis of these hybrid cell DNAs with molecularly cloned nucleic acid probes permits the chromosomal, and sometimes subchromosomal localization of human genes. Previous studies have resulted in the chromosomal mapping of the p53 cellular tumor antigen gene, ten protooncogenes, kappa and lambda light chain and heavy chain immunoglobulin genes and pseudogenes, the α, β, and Y fibrinogen genes, calcitonin, collagen type III, J protein gene and a processed pseudogene, P_1 450 cytochrome oxidase, the metallothionein multigene family on five chromosomes, a Diffuse Lymphoma transforming gene, and a transcriptionally active translocation breakpoint, BCL2, in Follicular Lymphoma. Recent studies have localized human β-polymerase on chromosome 8 (8pter-q22) and identified a DNA polymorphism in the 5' region of the gene which will be useful in evaluating the role of this

gene in diseases involving defective DNA repair. A putative α-polymerase cDNA identifies a family of hybridizing sequences and it appears to map on the X chromosome short arm (Xp). A dioxin-inducible $P_3$450 and a debrisoquine-inducible P450 gene were mapped to chromosomes 15 and 22, respectively. A poly-ADP Ribose synthetase 1.2 kb cDNA fragment identifies three hybridizing sequences located on chromosomes 1q, 13, and 14 or 15; it has not been determined whether one, or all, of these loci is transcriptionally active. Genes for gastrin releasing peptide and N-acetylglucose-aminide β 1-4 galactosyltransferase and a low copy number sequence flanking satellite DNA have been mapped to chromosome 18q21, 9, and 4, respectively. Several useful restriction fragment length polymorphisms (RFLPs) have been identified with the various probes. Studies designed to establish genetic linkage maps of human chromosomes 1 and 15, and to localize a genetic defect in nevoid basal cell carcinoma syndrome by RFLP analysis of families have been undertaken.

Collaborative studies with Warren Evans on the GL-13 guinea pig leukemia model have revealed a transforming gene which was identified in the NIH 3T3 cell transfection assay. The resultant malignantly transformed mouse cells rapidly produce tumors upon injection into nude mice or syngeneic hosts. Guinea pig repetitive DNA sequences and a guinea pig N-ras gene have been identified in the primary and secondary transformant cells. Both the guinea pig normal and altered transforming N-ras genes have similar sizes. Hence, normal guinea pig bone marrow and leukemic DNA transformed mouse cell DNAs have each been size fractionated by Bulls Eye electrophoresis after EcoRI digestion; fractions enriched for guinea pig N-ras sequences are being cloned in EMBO λ phage vectors. Transforming activity of the cloned leukemic N-ras gene will be confirmed and alterations resulting in transforming activity will be identified.

B. Dr. Dean Hamer and coworkers: As a model for the regulation and function of eukaryotic genes, the metallothioneins (MTs) are being analyzed by molecular genetic and biochemical approaches. It was previously shown that a short DNA sequence present in multiple copies 5' to the mouse MT-1 gene is responsible for its inducibility by heavy metals. A mouse nuclear protein that interacts with the heavy metal control sequences of the mouse MT-1 gene has been detected by the exonuclease III footprint technique. It is regulated by cadmium in vitro and is being purified. A mouse cell nuclear extract capable of faithful transcription of the mouse MT-1 gene has been developed to compare regulatory sequence requirements for efficient in vitro and in vivo transcription and to assay nuclear regulatory binding proteins. The structural basis for differential metal binding by human MT-1 isoproteins has been investigated in isogenic cell lines by exon shuffling and gene transfer experiments. The results show that this effect is dominated by the carboxy terminal domain of the proteins, and identification of the precise amino acids involved is underway. Regulation of the MT-1 isoform genes during development and perturbation by activated H-ras oncogenes has been studied by introduction of cloned MT-I_E and MT-I_F fusion genes into normal and transformed human cell lines; preliminary results suggest that these two genes are repressed during development by fundamentally different mechanisms.

In yeast, trans-acting mutants that alter metallothionein gene regulation have been isolated. These mutations are being mapped and the corresponding genes isolated by complementation. A substantial number of mutants have been engineered into the yeast metallothionein coding sequence to determine the role

of individual amino acids in the structure and function of the protein. The 54 amino acid yeast protein is generated by cleavage of an 8 amino acid peptide from the NH_2-terminus. Mutants affecting the NH_2-terminus were constructed and tested with the following results: changes in residues 2 and 3 did not affect normal cleavage; changes in residues 8 and 9 completely blocked cleavage at the normal site; deletion of residues 2 to 8 resulted in synthesis of essentially mature protein without cleavage. None of the mutations effected the ability of metallothionein to chelate copper in vivo or in vitro. Immunofluorescent microscopy with an antibody prepared against yeast MT demonstrated a cytoplasmic localization for both the wild-type and mutant MT proteins. Mutants have also been designed to test the copper-binding cysteine residues of the protein. Four different truncations of the molecule have been engineered each introducing a corresponding double cysteine change. Preliminary studies indicate that these alterations decrease the copper binding properties of the protein in vivo and in vitro with the different mutations exhibiting different quantitative effects.

IV. DEVELOPMENTAL BIOCHEMISTRY AND GENETICS (Dr. Michael Yarmolinsky, Chief)

The Developmental Biochemistry and Genetics Section is made up of three independent research groups.

A. Dr. Michael Yarmolinsky and coworkers: The group seeks to understand the replication and partition mechanisms that account for the stable inheritance of unit-copy bacterial plasmids. The P1 plasmid prophage chosen for study is representative of a class of genetic elements characterized by the presence of iterated DNA sequences at the origin of plasmid replication and in a replication control region. It is also characterized by a specialized partition apparatus. In the period of this report the laboratory has provided conclusive evidence for the existence of a novel biological control mechanism in which the control region acts in trans without mediation by messenger RNA. Means of testing the validity of alternative schemes by which this control mechanism might work have been devised. The group is also interested in the roles played by alternative origins in P1. From P1 phages that cannot replicate from their normal dnaA-dependent origin, insertion mutants which fail to replicate from an alternative dnaA-independent replicon have been obtained. Surprisingly the phage is still proficient for replication, indicating the presence of yet another origin. Preparatory to the isolation of bacteria with altered capacity for plasmid partitioning, λ-miniP1 hybrids have been constructed bearing markers that permit selection, counterselection and visual discrimination of mutants.

B. Dr. Carl Wu and coworkers: Using a new exoIII footprinting assay, the group has purified Drosophila heat shock gene activator protein to homogeneity. The protein migrates with a mass of 110 kilo daltons on denaturing gels; its binding activity is detectable within minutes of heat shock. The binding occurs with high affinity ($K_D = 4 \times 10^{-12}M$) to the heat shock control sequence. An exoIII footprinting technique developed by the group has been applied successfully to the Drosophila heat shock gene TATA box sequence, and to the rat insulin II gene and the Drosophila segmentation gene fushi tarazu.

C. Dr. Bruce Paterson and coworkers: This group is concerned with the mechanisms governing the expression of specific genes in differentiated cells. They have demonstrated that the level of expression and the appropriate regulation of the various chicken actin genes, when stably introduced into mouse

myogenic cell lines, is dependent upon the developmental history of the particular line. The skeletal actin genes are not appropriately regulated in adult myogenic satellite cells, whereas correct regulation is observed in embryonically derived lines. The cytoplasmic beta actin down regulates during myogenesis in all muscle lines tested so far. These various lines were used to demonstrate that all the sequence information necessary for the regulated expression of the actin genes was present in the genomic isolates tested. Promotor constructs with the bacterial CAT gene have shown that the transcriptional control and regulatory regions for cardiac actin are contained within the 300 bp 5' to the start of transcription. Transcription of the beta cytoplasmic actin is not directly regulated through the promotor region, as determined by promotor exchanges and CAT constructs. Deletion analysis has defined a 320 bp region spanning the polyadenylation signal that is responsible for the transcriptional regulation of beta actin. The promotor regions for the myosin LC1/LC3 gene have been defined with CAT constructs; sequences involved in the regulated, tissue specific expression of these genes are contained within the promotor regions. Two lower eukaryotic system are under investigation: the cytoplasmic myosin gene of Acanthamoeba and the myosin gene of yeast. The group isolated and determined the nucleotide sequence of the Acanthamoeba gene and compared its structure and organization to other myosin genes. Using a probe from the myosin gene of C. elegans they identified homologous sequences in the yeast genome.

PC12 cells undergo neuronal differentiation in response to nerve growth factor (NGF). The group has isolated a cDNA sequence and the corresponding gene that is induced 50-80 fold in response to NGF. The cDNA encodes a polypeptide of 72,000 daltons. Antibodies have been made to different portions of the cDNA using lacZ fusions and are being used to study the induction and localization of the protein. The promotor of the NGF induced gene responds to NGF when fused to CAT and transfected into PC12 cells.

IV. MACROMOLECULAR INTERACTIONS SECTION (Dr. Claude Klee, Chief)

This Section is composed of three independent groups.

A. Dr. Cary Queen and coworkers: This group has continued to study the regulation of expression of the immunoglobulin gene family. They previously showed that a cloned kappa immunoglobulin gene is transcribed after transfection into antibody producing myeloma cells but not in non-lymphoid cells or L cells. Thus, specific cells can appropriately regulate the kappa gene even when not in its normal chromosomal environment. By deleting different parts of the cloned gene, they first showed that an enhancer element, downstream of the promoter, is necessary for the specific transcription of the gene in myeloma cells. They have now demonstrated that the promoter also plays a role in cell specific regulation of transcription of the immunoglobulin gene. The kappa enhancer stimulates the kappa promoter 20 fold more than it stimulates other promoters. The synergism between the kappa enhancer and promoter suggests the existence of a cell specific protein which recognizes both regulatory elements.

B. Dr. Paul Wagner and coworkers: This group is mainly concerned with the regulation of cellular motility. The focus of their attention is the mechanism by which acto-myosin drives motile activity in non-muscle cells. During the last two years they have made two major observations. Whereas in avian smooth muscle, phosphorylation of the regulatory light chains is believed to act as an

on-off switch of the actin-activated ATPase of myosin, Wagner and his colleagues have shown that phoshorylation of the light chains of thymus or mammalian smooth muscle is only involved in the modulation of actin-myosin interaction. Second, at saturating actin concentrations, non-phosphorylated avian gizzard myosin is inactive, both phosphorylated and non-phosphorylated thymus or mammalian aorta myosins are fully active. Therefore, regulation of motility in avian smooth muscle and mammalian non-muscle and smooth muscle cells is different. These conclusions were validated by measurements of the myosin ATPase. Nonphosphorylated aorta or thymus myosin, but not unphosphorylated gizzard myosin, induce contraction of myosin-depeleted skeletal muscle myofibrils. Thus, in addition to myosin light chain phosphorylation, mammalian non-muscle or smooth muscle cells may contain an alternative mechanism to regulate actomyosin based motility by an alternative mechanism.

C. Dr. Claude Klee and coworkers: The major goal of this group is to understand the mechanism of stimulus-response coupling mediated by calcium or cAMP acting as second messengers. Three different aspects of this problem were studied this year. (1) It was shown that the inositoltriphosphate-induced calcium release from the endoplasmic reticulum is modulated by intracellular calcium levels. (2) At physiological concentrations, magnesium modulates the interaction of calmodulin with calcium and therefore plays a role in the regulation of cellular responses by calmodulin. Only in the presence of magnesium, is a large difference seen between the affinities for calcium of sites III and IV, located in the carboxyl-terminal half of calmodulin, and site I and II in the amino-terminal domain. This difference, together with the positive cooperativity previously observed, explains the stepwise nature of the conformational changes induced by calcium. It was previously shown that different calmodulin-regulated enzymes recognize different domains of the calmodulin molecule and may therefore be activated by different calmodulin conformers. Thus, the stepwise changes exhibited by calmodulin, at different calcium levels, can be used to regulate different metabolic pathways. (3) The gene of the catalytic subunit of cAMP-dependent protein kinase of Drosophila melanogaster has been cloned and will be used to study the role of cAMP-dependent phosphorylation in this organism.

VI. NUCLEIC ACID ENZYMOLOGY (Dr. Maxine Singer, Chief)

The work of this Section is directed toward an understanding of the structure and possible function of highly repeated DNA sequences and to the elucidation of the mechanisms by which such sequences are amplified either in tandem or through transposition to new genomic loci.

The extraordinary polymorphism of satellite DNA and the fact that no function can be associated with these sequences lead these investigators to suggest that they might be a by-product of DNA replication or recombination in localized chromosomal regions. Therefore, efforts have been directed toward characterizing the DNA sequences in the vicinity of satellites. An apparently single-copy DNA sequence that neighbors satellite DNA was cloned from the African green monkey genome. Analysis of genomic libraries from human and mouse indicate that both the single-copy sequence and its association with species-specific satellite DNA are conserved in mammalian DNA. However, in spite of the substantial evidence that the sequence is single-copy in monkey and human DNA, two types of cloned genomic segments are obtained from these two organisms only

one of which is colinear with the genomic arrangment of the sequences as characterized by DNA-blotting. Although other interpretations remain possible, current data are most consistent with the idea that the cloned segments undergo rearrangements during cloning in E. coli when both the satellite and the single-copy sequence are present together in a lambda phage vector. The single-copy sequence has been localized on human chromosome 4. In other studies, the remarkable evolutionary fluidity of satellite DNA is being used to analyze the evolutionary and taxonomic relations among the carnivores in the felid and canid families.

During this year, the work on SINES has focused on two aspects. First, a SINE family unique to carnivores has been isolated and characterized from Felis catus; the organization of the family SINE can be used to distinguish carnivore genera and species. Second, cloned ALU sequences (the typically primate SINE family) have been used to detect and purify sequence-specific binding protein in nuclear extracts from primate cells. Exonuclease III protection studies indicate that the binding site is located downstream of the A-box sequence associated with the RNA polymerase III promoter found on ALU sequences.

Previously this group reported that polyadenylated, cytoplasmic transcripts of the LINE-1 family are detectable in the human teratocarcinoma cell line, NTera2D1 and that these RNA's represent the strand containing long open reading frames. Primer extension studies have now indicated that the bulk of the RNA initiates at the position previously identified as the first common base in full length primate LINE-1 units. This finding is consistent with the proposal that the bulk of genomic LINE-1 sequences are processed pseudogenes and may arise through reverse transcription. Approximately 20 cDNA clones isolated from a library constructed with the NTera2D1 cytoplasmic RNA were partially characterized. The lengths of the cDNA's vary from about 4.5 to about 6 kbp and no two of the cDNA's are identical. Thus, the RNA's appear to represent a sub-set of closely related LINE-1 units that are distinguished from the majority of genomic LINE-1 units by being associated with regulatory elements that account for their specific transcription in the teratocarcinoma cells. In addition, the cDNA's have a common consensus sequence in the 3'-untranslated region that is different from the consensus sequence of randomly isolated genomic LINE-1 elements. The majority of the 20 messenger RNA's represented by the cDNA's can not encode a protein corresponding to the 4-5 kbp of coding sequence that is highly conserved in the LINE-1 families of different mammals; single base pair changes result in stop codons. Six of the 20 cDNA's do have completely open reading frames in the 2500 base pairs sequenced thus far. Additional sequence data in the 5'-half of the cDNA's is being obtained in order to analyze the full extent of the open reading frame in these cDNA's.

VII. PROTEIN CHEMISTRY (Dr. Elbert Peterson, Chief)

The Protein Chemistry Section is made up of three independent research groups.

A. Dr. Michael Mage and coworkers: This group, in collaboration with Dr. L. Romani of the University of Perugia, has investigated a possible requirement for Class II major histocompatibility complex (Ia) molecules (either on syngeneic accessory cells or on allogeneic stimulators) in the initial activation of cytotoxic T lymphocyte precursors (CTLp) for allocytotoxic responses.

Earlier experiments had indicated that such cells were best triggered into mature CTL in vitro by cells known to be Ia^+. However, experiments in which high purified C57BL/6 $Lyt\text{-}2^+$ splenocytes were co-cultured with P815 stimulator cells and interleukin 2 have now shown that the early activation of CTLp is independent of Ia^+ syngeneic accessory cells.

"Xenogenization" (induced mutation in tumor cells to increase their immunogenicity) is a potential approach to cancer immunotherapy of tumors that fail to elicit an effective immune response. In a further collaboration with Dr. Romani, the humoral response in mice to a chemically xenogenized murine lymphoma cell line (identified last year only through its ability to elicit a cellular immune response) was characterized. Antibodies of both IgG and IgM classes capable of binding the xenogenized tumor could be detected after a single sensitization and reached a maximum after 3-4 injections, when most of the humoral response was made up of antibodies of the IgG class. Cross reactivity with the parental line was completely removed by absorption of the hyperimmune serum on parental cells, which removed most of the IgM antibodies. The novel determinants recognized on the xenogenized cells were not close to those shared with the parental tumor since saturation with anti-parental antibodies did not prevent subsequent binding of the hyperimmune absorbed serum. The latter did not cross-react with normal hemopoietic or lymphoid cells of the same or allogeneic haplotypes.

Dr. Mage is also developing another approach to the immunotherapy of tumors: The immunochemical attachment of antigens to tumor cells in order to increase their susceptibility to cellular immune reaction. Antibodies to public specificities of class I molecules are used to attach allogeneic affinity-purified molecules to mouse lymphoid tumor cells. Class I major histocompatibility complex molecules were chosen for initial experiments because of their strong immunogenicity in cellular immunity. Using purified mouse and rat monoclonal antibodies, he has not been able to demonstrate attachment of allogeneic class I molecules to the cell surface, but rabbit polyclonal anti-mouse class I MHC antibody does successfully bind allogeneic class I molecules to the cell surface. Procedures for the effective use of the monoclonal antibodies are being sought, and the use of class I antigen-coated tumor cells as immunogens and targets for cellular immunity is being studied.

B. Dr. Warren Evans and coworkers: Last year these investigators reported the isolation in highly purified form of a factor in guinea pig serum that stimulates the maturation of neutrophil precursors from guinea pig bone marrow. This maturation inducing activity, initially named "myeloblast maturation factor" (MMF), and now called "neutrophil precursor maturation factor" (NPMF) is associated with transferrin. In collaboration with Dr. Michael Mage, monoclonal antibodies are being made, and several anti-NPMF positive clones have been obtained.

Studies in collaboration with Drs. Jana Bednarek, Robert Knight and Grant Taylor of the Walter Reed Army Hospital are under way to develop an in vitro maturation assay for human neutrophil precursors corresponding to that developed for guinea pig cells. Using marrow from normal human donors, viable neutrophil precursors can be isolated in sufficient yield to permit 25-30 assays per marrow sample. In the presence of dialyzed human serum, neutrophil precursors undergo maturation to mature end stage cells in 5-6 days. In contrast, cells in cultures containing normal human serum albumin as a protein control form mainly

macrophages under the same conditions. As in the guinea pig system, the maturation of human neutrophil precursors in the presence of human serum was accompanied by a pronounced increase in alkaline phosphatase activity. This enzyme has been shown to occur, in bone marrow, only in the late stage of maturation of neutrophils. The serum effect on both morphological maturation and alkaline phosphatase activity is enhanced 50 to 60% by the addition of zinc sulfate (10^{-5}M) to the culture medium.

This group is also developing a method for the early detection of blast crisis in CML patients. Using the combined HPLC-SDS gel electrophoresis procedure previously reported, five CML patients have been followed for several years from the stable phase to the blast crisis stage of the disease. The results indicate that prior to the onset of blast crisis there is a progressive decrease in both cell membrane and granule phenotypic proteins in mature granulocytes.

In collaboration with Drs. O.W. McBride, an attempt is being made to identify the genes in CML myeloblasts that are responsible for leukemogenic activity (see above).

C. Dr. Elbert Peterson: The work on the ion-exchange displacement chromatography of proteins, using carboxymethyldextrans (CM-Ds) as displacers continues. In collaboration with Dr. Makonnen Belew of the University of Uppsala, it has been found that the addition of very small amounts of sodium borohydride to the reaction mixtures during the synthesis of the displacers suppresses the formation of groups that absorb light at 280 nm. Thus ordinary, otherwise unprotected dextran can be used at higher temperatures and with higher concentrations of reactants without the generation of UV absorbance that interferes with UV monitoring of chromatographic profiles. In this way, the necessary range of substitution was achieved at 38° or 50°C with approximately half as much reagent, and the background A_{280} was about one fifth of that of unprotected products prepared at room temperature. Consequently, about 25 to 60 g of CM-D(Na), depending on the level of substitution selected, can be purified with resin columns totaling 270 ml. The columns can be regenerated in situ for reuse; the present set has been used more than 40 times so far, with no sign of diminishing performance.

Although narrow-range CM-Ds obtained by displacement fractionation of the purified CM-D are required for the very high resolution of proteins that was demonstrated in earlier work, recent experiments have shown that much can be accomplished with the unfractionated CM-Ds that are obtained directly from the purifying resin columns with relatively little investment of labor and time. Thus, a protein mixture can be fractionated by applying it to a suitable anion exchanger and following it with several column volumes of a dilute solution of unfractionated CM-D before the final displacer is begun. Two or more of the unfractionated CM-D preparations can be used in a single mixture to provide more uniform spacing.

The unfractionated CM-D preparations can also be mixed in small quantity with the protein sample to promote protein-protein displacement as the sample enters the column, so that the target protein can be obtained in a highly enriched fraction with very large sample loads.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00333-23 LB

PERIOD COVERED
October 1, 1985, to September 30, 1986

TITLE OF PROJECT *(80 charactars or less. Title must fit on one line between the borders.)*
Biochemical Basis for Defective Differentiation in Granulocytic Leukemia

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

W. H. Evans	Research Chemist	LB	NCI
E. A. Peterson	Chief, Protein Chemistry Section	LB	NCI
S. Wilson	Biologist	LB	NCI
M. Mage	Immunochemist	LB	NCI
W. McBride	Chief, Cellular Regulation Section	LB	NCI
R. Balachandran	Visiting Associate	LB	NCI

COOPERATING UNITS *(if any)*
Hematology, Oncology Section, Walter Reed Army Medical Center

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 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 external cell regulators on granulocyte differentiation, as measured by changes in the synthesis of specific cellular components, are studied in a defined culture system previously developed in this laboratory. Possible relationships between transforming genes in leukemic myeloblasts and factors involved in the regulation of normal granulocyte differentiation are under investigation.

Project Description

Methods Employed:

Leukocytes are isolated from bone marrow, blood and inflammatory exudates by a variety of methods. Subcellular fractions are prepared by differential centrifugation. Proteins extracted from leukocytes and serum proteins are fractionated by column chromatography, high performance liquid chromatography (HPLC), and gel electrophoresis. Glycolipids are separated by thin layer chromatography and analyzed by gas-liquid chromatography and mass spectrometry. Immunological reagents, including fluorescent antibodies, are prepared against granulocyte proteins and used to follow their formation during granulocyte maturation. Stationary tissue culture methods are employed to study the synthesis of specific proteins from radioactive precursors. The distribution of radioactive proteins in electrophoretic gels is determined by slicing the gels in a gel fractionator and counting the slices in a liquid scintillation counter. Densitometric patterns of gels stained for glycoproteins are obtained by scanning the gels in a spectrophotometer equipped with a gel scanner accessory. Genetic analyses are carried out using transfection assays, agarose gel electrophoresis-Southern blot analyses of normal and leukemic DNA fragments obtained by restriction endonuclease cleavage and dot blot hybridization of cellular RNA with ^{32}P-labelled viral oncogene probes.

Major Findings:

A. Studies of the Neutrophil Precursor Maturation Factor (NPMF) in Guinea Pig and Human Serum.

We showed previously that the maturation of neutrophil precursors from guinea pig bone marrow is dependent on a factor [previously named myeloblast maturation factor (MMF) and now called NPMF] in guinea pig serum that closely resembles transferrin in its physical and immunochemical properties. A paper describing this work has been accepted for publication in Leukemia Research. Studies in collaboration with Dr. M. Mage are in progress to further test the homogeneity of this factor and to assess its binding to normal and leukemic neutrophil precursors. For this work we are attempting to prepare a monoclonal antibody against NPMF. Mice immunized with highly purified NPMF have been used to prepare mouse spleen-myeloma cell hybridoma cultures in which antibodies to NPMF have been detected. Cloning of these hybridoma cells is now in progress and several anti-NPMF positive clones have been detected.

Studies in collaboration with Dr. Jana Bednarek, Dr. Robert Knight and Dr. Grant Taylor of the Walter Reed Army Hospital, are now in progress to develop an *in vitro* maturation assay for human neutrophil precursors similar to that developed for guinea pig cells. We find that, using marrow samples obtained from the iliac crest of normal donors, viable neutrophil precursors can be islolated from these samples by Ficoll density centrifugation in sufficient yield to permit up to 25-30 assays per marrow sample. Using the cell culture system developed for studying neutrophil precursors from guinea pig marrow, we find that, in the presence of dialyzed normal human serum, neutrophil precursors undergo maturation to mature end stage cells in 5-6

days. In contrast, cells in cultures containing normal human serum albumin as a protein control form mainly macrophages during this time period. As in the guinea pig system, the maturation of human neutrophil precursors in the presence of human serum showed a pronounced increase in alkaline phosphatase activity as the cells matured. This enzyme has been shown to be absent in the cells of the bone marrow except for cells in the neutrophil series where it is found only in the late stage of maturation. We also find that the serum effect on morphological maturation and alkaline phosphatase activity is greatly enhanced (50-60%) by adding zinc sulfate (10^{-5}M) to the culture medium. Screening of serum chromatographic fractions for NPMF activity is now underway using alkaline phosphatase activity as a marker of maturation induction in these cultures.

Preliminary studies of leukemic blast cells obtained from a patient with CML in blast crisis indicate that these cells fail to respond to the serum NPMF activity. These results are similar to those reported previously for leukemic blast cells from guinea pigs.

B. Molecular Basis of Defective Myeloblast Maturation in CML Blast Crisis.

For studies of the molecular mechanisms responsible for arrested myeloblast maturation in the blast crisis (BC) stage of CML we are using leukemic myeloblasts from the blast crisis stage of our guinea pig leukemia model for human CML. We are collaborating with Dr. O.W. McBride and Dr. R. Balachandran in an attempt to identify the genes in CML myeloblasts responsible for their leukemegenic activity. Studies thus far indicate that, unlike normal guinea pig DNA, DNA isolated from guinea pig leukemic blast cells is capable of transfecting NIH 3T3 cells and that these transfected cells are tumorogenic when transplanted into normal mice. Since evidence is accumulating that the N-ras oncogene might play a role in leukemogenesis in human CML we analyzed DNA obtained from second cycle transfected NIH 3T3 cells for the presence of guinea pig N-ras using the Southern blot technique. These studies show that transfection of NIH 3T3 cells is associated with the uptake of guinea pig DNA sequences including the N-ras ongocene. Studies are now underway to determine whether the cloned guinea pig N-ras oncogene has transforming activity in the NIH 3T3 cell assay.

C. Changes in the Phenotypic Protein Patterns of Mature Granulocytes Associated with the Transition from the Stable to the Blast Crisis Stage of Human CML.

Previously, we described a combined reverse-phase high performance liquid chromatography (HPLC)-SDS gel electrophoresis method for the study of the phenotypic protein patterns of mature blood leukocytes. Using this method, we have followed 5 CML patients over a period of several years from the stable phase to the blast crisis stage of the disease in an attempt to determine whether any changes in the phenotypic protein pattern of mature granulocytes preceeds or accompanies the onset of the blast crisis stage of human CML. These studies indicate that prior to the onset of blast cirsis there is a progressive decrease in both cell membrane and granule phenotypic proteins in mature granulocytes. Other protein markers remain unchanged

during this transition and gradually become the predominant proteins in the phenotypic patterns of the mature cells. These residual unchanging proteins are also prominent in the phenotypes of the immature leukemic cells from the same patients. The development of this new phenotype in mature CML granulocytes appears to correlate with an increased aggressiveness and faster acceleration of the disease. These findings are supported by assays of mature cells from eight other CML patients who were diagnosed in the accelerated phase of the disease and expired in less than 12 months after the assays were performed.

Significance to Cancer Research:

Granulocytic leukocytes are produced in the bone marrow by a complex, multistage process of cell differentiation whereby proliferating, nonphagocytic, precursor cells are converted to nonproliferating phagocytic cells that play a crucial role in the body's defense against microbial invasion. Granulocytic leukemias can thought of as diseases in which this program for differentiation is arrested at various stages, resulting in the overproliferation of immature cells lacking, varying degrees, the capacity to carry out their normal function in the body's defense against infections. At present, the mechanisms involved in the initiation and modulation of the various steps in the differentiation program are poorly understood. Our research is aimed at identifying the humoral factors and clarifying the molecular processes that control the appearance of specific subcellular components during granulocyte differentiation. Such information should be useful in developing therapeutic approaches for reversing the arrested differentiation of leukemic cells.

Thus far chemotherapy has not been able to delay the onset of the inevitable fatal blast crisis stage of human CML. Bone marrow transplantation during the chronic phase may hold the promise of a true cure for some CML patients since the malignant clone appears to be permanently eliminated. There has been a reluctance to initiate marrow transplantation therapy in the chronic stage since patients are usually symptom free and the duration of this phase can be unpredictably long. We are therefore attempting to develop methods for reliably predicting the onset of the terminal blast crisis stage of CML prior to the development of overt clinical symptoms so that marrow transplantation can be initiated at the earliest sign of this transformation. Optimal survival might then be achieved by allowing patients to be maintained in the chronic phase for as long as possible before more aggressive therapy is initiated.

Proposed Course of Research:

A. To isolate and purify the NPMF found in normal human serum and to determine whether the binding of this activity to human CML myeloblasts is abnormal relative to that of normal bone marrow myeloblasts.

B. In collaboration with Dr. Michael Mage we will attempt to isolate and purify the NPMF receptor in normal myeloblasts and determine whether or not this receptor is defective in leukemic myeloblasts. For this work we will use

isotope-labelled NPMF and antibodies to NPMF to detect the presence of NPMF receptor on intact cells or in various detergent lysates. If detected, this receptor will be purified using standard affinity separation procedures. We will then isolate the NPMF receptor from leukemic myeloblasts and determine whether it differs markedly in composition from the normal receptor. If the receptor mechanism involves a defective protein, then it would be important to determine whether the gene coding for this receptor is related to any of the known oncogenes. This working hypothesis for a possible mechanism of transformation in CML has a precedent in epidermal cellular transformation. In transformed epidermal cells, the receptor protein for epidermal growth factor (EGF) has been found to be defective and the defect has been related to a known oncogene (V-erb-B) which codes for the EGF receptor.

C. The availability of our guinea pig model for human CML opens up many new possibilities for studies of the molecular genetics of this disease, especially since some of these studies cannot be carried out in humans. Studies in collaboration with Dr. O.W. McBride will be aimed at cloning the guinea pig N-ras oncogene from DNA obtained from NIH 3T3 cells transfected with guinea pig leukemia myeloblast DNA. The corresponding gene from normal guinea pig myeloblasts will also be isolated and compared to the N-ras oncogene from leukemic cells for transforming capacity in the NIH 3T3 cell transfection assay.

In human CML the Abelson oncogene is translocated from the number 9 to the number 22 chromosome. This translocation is the paradigm of the consistent chromosomal change associated with a specific type of malignancy (i.e. CML) but the role played by this specific translocation in the pathogenesis of the disease is still unknown. Recent studies in Dr. Annelies DeKlein's laboratory at the University of Rotterdam, suggest that the conjunction of the breakpoint cluster region (bcr) on chromosome 22 and the c-abl translocation constitute a gene rearrangement which could play a fundamental role in the pathogenesis of CML. We are now collaborating with Dr. DeKlein's group in studies in which human and rodent bcr probes are being used in Southern blot analyses of DNA obtained from our guinea pig leukemic myeloblasts to determine whether c-abl undergoes translocation and alteration in a manner similar to that observed in human CML.

D. To further develop the use of HPLC as a method for phenotypically characterizing the degree of differentiation of purified populations of normal and leukemic granulocytes from guinea pigs and humans. This rapid screening method might be useful in classifying or staging AML and CML patients for various types of therapy.

E. To investigate various potential agents for inducing leukemic cells to mature either partially or completely in vitro. Such agents could serve as attractive adjuncts, or in some cases, as alternatives to cytotoxic therapy, for they would inhibit the expansion of the leukemic population while leaving normal granulocytes unharmed.

Publications:

Evans, W.H., Bednarek, J.M., Alvarez, V.L., Wright, D.G., Terebelo, H.R., and Taylor, G.: Direct analysis of differentiation proteins in normal and leukemic human granulocytes by high performance liquid chromatography. J. Natl. Cancer Inst. 75: 227-235, 1985.

Evans, W.H., Wilson, S.M., and Mage, M.G.: Transferrin induces maturation of neutrophil granulocyte precursors in vitro. Leukemia Res. (in press).

Appendix: NIH Contract No. 1-60-25423

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

Man Years Purchased: 1

Major Findings:

During the past year this contract was used primarily for the in vivo and in vitro maintenance of cells from our guinea pig model for human CML. These cells were also used for our studies aimed at identifying the molecular mechanisms responsible for arrested myeloblast maturation in the blast crisis stage of CML.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00366-16 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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
A. Feenstra	Visiting Fellow	LB	NCI
Z. Grossman	Visiting Fellow	LB	NCI
P. Arnaud	IPA	LB	NCI

COOPERATING UNITS *(if any)*
E. Leiter, Jackson Laboratory, Bar Harbor, ME; K. Moore, DNAX Research Institute, Palo Alto, CA; K. Ishizaka, Johns Hopkins School of Medicine, Baltimore, MD; Laboratory of Biology, NCI

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Biosynthesis Section

INSTITUTE AND LOCATION
National Cancer Institute, 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

B

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

We have continued studies of the intracisternal A-particle (IAP) genes, a genetically distinctive type of retrovirus-like element or retrotransposon that is extensively reiterated in the genomes of mouse and other rodent species. The family of mouse IAP elements, about 1000 per haploid genome, including a number of structural variants with deletions and/or insertions at characteristic positions. Last year, we described a collaborative study with Drs. K. Moore and K. Ishizaka in which a cDNA clone encoding an IgE-binding factor (a form of T-cell-secreted IgE-regulatory protein) was identified as a member of the IAP gene family. We have since characterized 5 additional cDNA clones that code for IgE-binding factors. Each one proved to be a different structural variant of the full-length IAP proviral element. Antisera against the IAP core protein 73 reacted with the IgE-binding factors, and isolated p73 itself was shown to bind to IgE and to potentiate IgE production in immunized B-lymphocytes. Dr. Z. Grossman has isolated several cDNA clones from normal mouse thymus by immuno-selection for the production of proteins cross-reactive with p73. These clones are being compared with the IgE -binding factor clones by sequence analysis. J. Mietz has essentially completed the nucleotide sequencing of MIA14, a full-size 7.3 kb mouse IAP genomic element. In other IAP-related studies, Dr. A. Feenstra completed work showing that in vitro DNA methylations at a cluster of three HhaI sites in the 5' region of a cloned IAP LTR completely abolished the capacity of the LTR to promote CAT gene expression in an appropriately constructed recombinant plasmid; and that methylation at a single HpaII site very near the 3' end of the LTR reduced activity by 75%. These observations define a system for elucidating the mechanism by which methylation inhibits IAP and other retroviral expression. J. Fewell has identified as hnRNP proteins A1 and A2 the nuclear antigens that cross-react with an antiserum prepared against the IAP core protein p73. He has shown that these antigens can be markedly depleted under certain growth conditions and is examining the possibility that proteins A1 and A2 are produced in rate-limiting amounts in rapidly growing cells.

Project Description

Objectives:

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

Methods Employed:

Culture of animal and bacterial cells; recombination and cloning of specific eukaryotic and viral DNA sequences in plasmids, lambda phage and retrovirus vectors; analysis of DNA components by restriction endonuclease cleavage, electrophoresis, and blot hybridization; chromosomal localization of specific DNA sequences by *in situ* hybridization; electron microscopy of DNA hetero-duplexes; nucleotide sequencing by the chemical procedure of Maxim and Gilbert and the dideoxy method of Sanger; chromatographic and electrophoretic analysis of cellular and viral proteins; detection of specific antigens (proteins) "Western" blots; radio-immunoassay and immunoprecipitation of specific antigens; immuno-fluorescent staining; transfection of cultured cells with recombinant plasmids formed from expression vectors.

Major Findings:

Intracisternal A-particle (IAP) genes represent a distinctive class of endogenous retrovirus-like elements that are extensively reiterated in the cellular DNA of *Mus musculus* and certain other rodent species. The family of mouse IAP genes, about 1000 per haploid genome, includes structural variants with deletions and insertions at characteristic positions. During the past year we have continued studies on the properties and functional roles of peptides produced by full-sized and variant genes, on the number and identity of IAP genes expressed in normal mouse thymocytes, on the functional properties of IAP LTRs as related to CpG methylation, on the possible role of IAP expression in a genetically determined form of mouse diabetes, and on the identification of a nuclear antigen detected by antiserum prepared against IAP structural protein.

A. IAP Genes and IgE-binding Factor (IgE-BF)

Last year we described collaborative studies with groups at DNAX Research Institute (Dr. K. Moore and coworkers) and at Johns Hopkins Medical School (Dr. K. Ishizaka and coworkers), in which a cDNA clone encoding an IgE-BF was identified as member of the IAP gene family. IgE-BF are immunoregulatory proteins secreted by normal T-cells and by certain T-cell hybridomas. These proteins bind specifically to the FcE portion of rodent IgE's and, according to their glycosylation state, can potentiate or suppress IgE production in differentiating B-cells. Factors with identical properties are produced by human T-cells.

The primary translation product of the cDNA clone (clone 8.3) was identified as a 60 kDA IAP-related gag-pol fusion protein. Some of this protein was processed to an 11 kDa peptide. Both the 60 and 11 kDa products had identi-

cal properties of IgE binding and potentiation of the IgE response. We showed that clone 8.3 formed heteroduplexes throughout its entire length with an authentic mouse genomic IAP element (MIA14) and that clone 8.3 and MIA14 were 90-95% homologous in nucleotide sequence over the region coding for the 11 kDa moeity. Antibodies against the IAP gag protein (p73) bound both the 60 and 11 kDa IgE-BF encoded by clone 8.3, the IgE-BF produced by the hybridoma from which clone 8.3 was prepared, and the IgE-BF produced by normal rat and mouse T-cells.

During the past year we characterized 5 more cDNA clones that code for IgE-BF. Each one proved to be a different structural variant of the full-length 7.3 kDa IAP proviral element. Information coding for IgE binding proteins is apparently part of the IAP gag-coding sequence. Antisera prepared against oligopeptides specified by the sequence of clone 8.3 were shown to immunoprecipitate IAP proteins synthesized by several different cell types. Dr. Ishizaka has found authentic IAP p73 to contain components with IgE-binding and IgE-potentiating effects. We are trying to identify the active protein species. This may represent a small subset of the whole, since biological activity of IgE-BF is dependent upon glycosylation of the factors but the p73 fraction of isolated IAPs is known to contain minimal levels of carbohydrate.

B. Isolation of IAP cDNA Clones from RNA of Normal Mouse Thymus

Last year, we reported that IAP transcripts and IAP-specific proteins were present in mouse thymus. The levels of IAP gene products varied characteristically among a number of different mouse strains examined. This year, Dr. Z. Grossman has prepared cDNA libraries in the lambda gtll expression vector from thymus polyA-RNA of three mouse strains, and has isolated clones encoding gal-fusion proteins that immunoreact with antiserum against p73. These clones are presently being analyzed.

C. Nucleotide Sequencing of a 7.3 kb Genomic IAP Provirus-like Element

J. Mietz has essentially completed the sequencing of mouse genomic clone MIA14. This element is a pseudogene in all of the coding regions. Nevertheless, by reference to the reported sequence of a complete hamster IAP element (also a pseudogene) and to the protein coding sequences from the IgE-BF cDNA clones, the MIA seqence serves to delineate the specific mouse IAP genetic regions and provides a basis for comparisons between these and other classes of retrotransposons.

D. Effects of In Vitro Methylation on the Promoting Activity of a Cloned IAP LTR

IAP genes are known to be heavily methylated in normal cells and tissues, and to be undermethylated in cells actively producing IAPs. Also, IAP production can be significantly enhanced by treatment of cells with 5' azacytidine, an agent known to inhibit DNA methylases. A favorable distribution of methylation-sensitive restriction sites in the LTR of MIA14

permitted Dr. Feenstra to examine the effects of site-specific methylations on the promoter activity of the LTR coupled to the CAT gene and transfected into COS7 cells. Promoter activity was completely abolished by methylation of three HhaI sites clustered in the 5' portion of the LTR, and reduced by 75% upon methylation of a single HpaII site near the 3' end. The system defined by this study provides a means to elucidate the mechanism by which methylation regulates the activity of IAP genes and, by extension, the activity of other integrated retroviruses whose activity is suppressed in a similar fashion.

E. IAP Gene Expression as a Possible Factor in the Physiopathology of a Genetically Determined Form of Mouse Diabetes

We have previously reported collaborative studies with Dr. E. Leiter of Jackson Laboratory showing that IAP gene products are expressed at higher levels in the pancreatic beta cells of inbred mice susceptible to the db gene than in the beta cells of resistant strains. We postulated that IAP-related proteins on the beta cell surface might act as neoantigens to induce an autoimmune attack and cell necrosis. Several years ago, I observed that normal mice of several inbred strains carried appreciable titers of anti-p73 antibody activity, as measured in ELISA with p73 as the immobilized antigen. Dr. Leiter has followed this up and has found that titers are low in the first 8-10 weeks of life and increase rather abruptly thereafter to adult levels. This time course would be compatible with a role for the circulating antibodies in the generation of beta cell necrosis. However, Dr. Leiter has found that mice carrying both the db and scid (severe combined immunodeficiency disease) genes in homozygous form develop typical beta cell necrosis and diabetes in spite of the complete absence of circulating antibodies. This observation indicates that any response mounted against IAP or other surface antigens must be effected at the cellular level, presumably by macrophage activity since both B and T lymphocytes are absent in the scid/scid animals. It is possible that IAP proteins do not have an initiating role in the disease process.

F. Identification of a Growth-dependent Nuclear Antigen Detected by an Antiserum Against IAP p73

One of our polyvalent rabbit antisera immunostains nuclei in a manner similar to sera from some autoimmune human patients. J. Fewell has found that the antigens responsible for this staining are much more abundant in confluent cultured cells (3T3, 3T6, NRK, BHK) than in sparse rapidly growing cells. In addition, the intranuclear distribution of the antigen undergoes striking changes during the cell cycle. Using A4.2 at silutions of 1/250 to 1/500 to detect antigen on Western blots. Mr. Fewell found that it reacts specifically with hnRNP protein components A1 and A2 from confluent 3T6 cells. These proteins can be depleted from the nucleus of sparse 3T6 cells grown under certain conditions. Antibody affinity-purified on electrophoretically isolated p73 retains its capacity to immunostain 3T6 nuclei and to react with A1 and A2 proteins. Dr. S. Wilson and coworkers have cloned the A1 gene (cDNA) from rat brain and expressed the protein in a bacteria system (see current Annual Report No. CB-05214-15). Antiserum A4.2 reacts with this product on Western blots.

Significance for Cancer Research:

IAP elements are endogenous retrotransposons which can act as insertional mutagens in both somatic and germ line cells of mice. IAP insertions have been shown to endow c-mos with transforming activity in a myeloma line and to activate an interleukin 3 gene in the myelomonocytic WEHI-3B cell line. In the latter instance, the insertion results in constitutive production of interleukin 3 and growth autonomy from exogenous T-cell-secreted factors. It is clear that transpositions of IAP elements can have important effects in tumor induction or progression.

Newly inserted IAP elements are generated by reverse transcription of IAP-specific RNA. It is likely that the frequency of insertional mutagenesis is correlated with the abundance and diversity of IAP gene expression. The factors that specify and regulate IAP transcription are therefore currently a major focus of our studies.

Proposed Course of Research:

A. We will continue collaborative studies to clarify the relationship between IAP genes and the IgE-binding factors.

B. The cDNA clones from normal mouse thymus will be characterized by restriction and sequence analysis, and compared to clones derived from T-cell lymphomas. We want to know whether additional IAP elements are activated in the transformed cells or whether constitutively active genes in the normal thymus are up-regulated (or both).

C. The sequencing of a complete IAP provirus-like element is essentially complete. The genetic organization will be analyzed and compared with other retroviruses and retrotransposons.

D. The detection and characterization of trans-acting binding factors that regulate the promoter activity of IAP LTRs will be actively pursued.

E. Additional studies on the possible role of IAP gene expression in the genesis of mouse diabetes will probably be minor.

F. The growth-related variation in hnRNP proteins A1 and A2 will be analyzed. The virtual disappearance of this proteins from certain rapidly growing cells suggests that they may be produced in rate-limiting amounts. The basis for the antigenic relationship between the IAP-p73 and these hnRNP components will be studied.

Publications:

Kuff, E.L., Fewell, J.E., Lueders, K.K., DiPaolo, J.A., Amsbaugh, S.C., and Popescu, N.C.: Chromosome distribution of intracisternal A-particle sequences in the Syrian hamster and mouse. Chromosoma 93: 213-219, 1986.

Leiter, E.H., Fewell, J.W., and Kuff, E.L.: Glucose induces intracisternal type A retroviral gene transcription and translation in pancreatic beta cells. J. Exp. Med. 163: 87-100, 1986.

Moore, K.W., Jardieu, P., Mietz, J.A., Trounstine, M.L., Kuff, E.L., Ishizaka, K., and Martens, C.L.: Rodent IgE-binding factor genes are members of an endogenous, retrovirus-like family. J. Immunol. (in press).

Feenstra, A., Fewell, J., Kuff, E.L., and Lueders, K.: In vitro methylation inhibits the promoter activity of an intracisternal A-particle LTR. Nucleic Acid Res. (in press).

Kuff, E.L., Mietz, J.A., Trounstine, M.L., Moore, K.W., and Martens, C.L.: cDNA clones encoding murine IgE-binding factors represent multiple structural variants of intracisternal A-particle genes. 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 00375-24 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

E.A. Peterson	Chief, Protein Chemistry Section	LB	NCI
M. Belew	Guest Researcher	LB	NCI

COOPERATING UNITS *(if any)*
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University of Uppsala, Sweden

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
National Cancer Institute, 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.)*

Methods for the fractionation and analysis of proteins are developed and applied to the purification of specific proteins for the study of their function and structure. Ion-exchange displacement (IED) chromatography is being developed for the fractionation of macromolecules and particles of biological interest, employing carboxymethyldextrans (CM-Ds) differing in number of charges per molecule as displacers. The procedure is particularly advantageous when large amounts of source material must be used to obtain sufficient amounts of a minor component, since the resolving power of the system can be focused on the narrow range of affinity represented by the protein of interest and its nearest neighbors. However, it is also applicable to ion-exchange HPLC. Recent efforts have been directed toward the simplification of the preparation of the displacers. Application of the method to the isolation of the myeloblast maturation factor (MMF) of W. Evans (Project Number Z01 CB 00333-22 LB) from guinea pig serum has shown that even unfractionated CM-Ds can be used to purify the factor. An exploration of the binding of serum proteins to several metal chelate and hydrophobic adsorbents indicates that chromatography on one or two of these before IED-chromatography can simplify the purification.

Project Description

Objectives:

To develop methods for the separation and analysis of proteins that will overcome present limitations and to apply these methods to the purification of specific proteins for the study of their functions.

Methods Employed:

Protein fractions separated by ion-exchange displacement (IED) chromatography are evaluated by gel electrophoresis, using both denaturing and nondenaturing conditions. Carboxymethyldextrans (CM-Ds) having a variety of degrees of substitution are prepared by reaction of dextran with chloroacetate in alkaline solution.

Major Findings:

A. The complexity of our earlier procedures for preparing carboxymethyldextrans (CM-Ds) for use as displacers has undoubtedly discouraged their exploration by other investigators and potential commercial suppliers. Our more recent, but as yet unpublished procedure employing organic ion-exchange resins to remove other reaction products prior to the fractionation of the CM-Ds into narrow-range products is much simpler. However, improvement in the efficiency of reaction was called for since the amounts of the reagents required to convert a given amount of dextran into CM-D determines the size of the resin columns needed to purify it. In the past, the temperature and reagent concentration that could be imposed during the reaction were limited by the need to suppress the formation of groups on the dextran that contribute significantly to absorbance of 280 nm by the final product, since the primary method for delineating the separated protein peaks in chromatography is based on their absorption of light at that wave length. In last year's report, this problem was overcome by the use of hydrogenated dextran from a commercial source. However, only dextran of about 5000 daltons was available in the hydrogenated form. This year a similar protection against the undesirable effects of higher temperature and more concentrated reagents has been extended to dextrans that have not been hydrogenated by adding very small amounts of sodium borohydride to the reaction mixtures. In this way, the necessary range of substitution was achieved at 38° or 50°C with approximately half as much reagent and the background absorbance of the CM-D was about one fifth of that of unprotected products prepared at room temperature. At present, about 25 to 60 grams of pure CM-D(Na), depending on the degree of substitution selected, can be obtained with resin columns totalling 270 ml. The columns can be regenerated *in situ* for reuse; the present set has been used 40 times so far, with no signs of diminishing performance.

Much of the increase in reaction efficiency has been achieved by reducing the water content of the reaction mixtures to the extent permitted by the need for effective mixing. Water, required as a solvent, is not only a

diluent but a reactant that competes with dextran for reaction with chloroacetate. Therefore, simple formulations relating water to the quantities of other reactants have been studied over the useful range of substitution, providing plotted data that permit the selection of reaction mixtures for preparing CM-D having any chosen affinity index.

B. Whereas narrow-range CM-Ds obtained by displacement fractionation of purified CM-D are required for the very high resolution of proteins that was demonstrated in our previous work, recent experiments have shown that much can be accomplished with the unfractionated CM-Ds that are obtained directly from the purifying resin columns with relatively little investment of labor and time. Since a given narrow affinity range is represented by only a small fraction of one of these very heterogeneous products, a substantial quantity must be used to provide adequate spacing of proteins over a wide range. For this reason, the separation of a protein mixture is carried out by allowing its components to become distributed within a modified frontal analysis of an unfractionated CM-D preparation. To accomplish this, the protein sample is applied to the column and followed immediately by several column volumes (e.g., 7 or 8) of a 0.5% solution of CM-D, which in turn is followed by a similar volume of final displacer. The protein components emerge from the column with varying degrees of separation. Unfractionated CM-D preparations having different affinity indices provide their best spacing in different regions of the chromatogram. When the desired protein has been located (by assay for its activity or by gel electrophoresis) in the fractions from a first experiment, a determination of the affinity indices of CM-D in fractions on each side of it will indicate which CM-D preparation should be used in the next experiment to optimize spacing. The use of a single mixture of two or more unfractionated CM-D preparations as a spacer in the first experiment is advantageous since such a mixture can be designed to provide a more uniform spacing over as wide a range of affinity as is likely to be needed.

Unfractionated CM-D preparations have been successfully applied to the purification of the myeloblast maturation factor (MMF) discovered by Dr. Warren Evans in normal guinea pig serum (Project number Z01 CB 00333-22 LB), even separating it from several apparently related proteins that are not resolvable by standard disc gel electrophoresis.

Certain proteins of this serum that have proved difficult to separate from the MMF in past experiments can be removed by initial passage of the serum through a hydrophobic column developed by Drs. J. Porath and M. Belew of Uppsala University.

The unfractionated CM-D preparations can also be mixed in small quantity with the sample to promote protein-protein displacement as the sample enters the column so that an anion exchanger can be greatly overloaded with respect to proteins lower in affinity than the target protein while allowing the latter (as well as more tightly adsorbed impurities) to accumulate on the column for subsequent displacement by another unfractionated CM-D preparation. In this way, a highly enriched fraction can be obtained with very large sample loads, and much less spacer CM-D is required.

In a continuing collaboration with Dr. A.R. Torres, a former co-worker in this laboratory now at Yale, the unfractionated CM-D preparations have been shown to be suitable for the separation of mixtures of model proteins (commercial preparations of ovalbumin, α-lactalbumin, and soy bean trypsin inhibitor) on a preparative scale, using commercial HPLC columns.

Significance to Cancer Research:

Displacement chromatography of proteins promises to be of value at any scale of operation and therefore has significance to all research that involves the isolation of such substances. The high capacity, resolving power, and convenience of these systems offer to expedite the recognition and isolation of minor protein components such as regulating factors and marker proteins of interest in disease. The anticipated development of a systematic, general procedure for the purification of nonhistone nuclear proteins that participate in the regulation of the transcription of genetic information would be of substantial significance to cancer research since defective control of these processes appears to be involved in cancer.

Proposed Course of Research:

Development of displacement systems for the separation of nonhistone nuclear proteins will be continued. A large scale isolation of HMG-1 and HMG-2 will be undertaken and the LMG proteins will be fractionated into several groups with selected narrow-range spacers.

Displacement chromatography will be applied to the purification of enzymes and other proteins of interest to members of this Laboratory.

Publications:

Belew, M., Peterson, E.A., and Porath, J.: A high-capacity hydrophobic adsorbent for human serum albumin. Anal. Biochem. 151: 438-441, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00945-13 LB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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
I. Oyamada	Visiting Fellow	LB	NCI
J. Palka	Visiting Fellow	LB	NCI

COOPERATING UNITS (if any): None

LAB/BRANCH: Laboratory of Biochemistry, DCBD

SECTION: Biosynthesis Section

INSTITUTE AND LOCATION: National Cancer Institute, NIH, Bethesda, MD 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.)

On the basis of previous experiments, we have proposed a model in which defects in collagen and proteoglycan synthesis in scurvy are caused indirectly by ascorbate deficiency. We suggested that the decreases were caused by changes in humoral factors during scurvy-induced fasting, rather than through the role of ascorbate in the biosynthetic pathway of either of these components. It had been assumed previously that defective connective tissue metabolism in this disease was due to defective proline hydroxylation and procollagen secretion. The results of our current studies provide additional support for our model. They show that there are changes in hormones and growth factors in the serum of scorbutic guinea pigs during the period when the animals are in a fasting state which are similar to changes in fasted animals and humans with no ascorbate deficiency. The exposure of normal cartilage chondrocytes to such scorbutic serum, with supplementation of ascorbate, resulted in decreased synthesis of extracellular matrix components. The collagen synthesized had normal levels of hydroxyproline. We conclude that the growth promoting activity of vitamin C in vivo is mediated initially through reactions other than those directly involved in connective tissue metabolism.

In a second study, we have found that the collagen phenotype of a 4-nitroquinoline-1-oxide transformed line of Syrian hamster embryo (SHE) fibroblasts, NQT-SHE, was markedly altered from that of normal SHE cells, which synthesized mainly type I procollagen, $(pro\alpha1)_2 pro\alpha_2$. Total collagen synthesis in the transformant was reduced to about 30% of the control level primarily because synthesis of one of the two subunits of type I procollagen, $pro\alpha1(I)$, was completely suppressed. The major collagenous products synthesized consisted of two polypeptides, designated as N-33 and N-50, which could be completely separated by precipitiation with ammonium sulfate at 33% and 50% saturation, respectively. Results of peptide mapping and other experiments indicate the N-collagens are altered $pro\alpha2(I)$ chains and that they differ from each other.

Project Description

Objectives:

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

Methods Employed:

A. Proteins of isolated tissues, cultured cells or cell-free systems are labeled with ^{3}H or ^{14}C-proline. The proteins are precipitated with trichloroacetic acid, redissolved and then assayed for radioactivity in collagen and noncollagenous proteins. The method involves specific digestion of collagen in a mixture of proteins using highly purified collagenase and was developed in our laboratory. The relative rate of collagen synthesis can be calculated from data obtained by this method and the specific rate of synthesis in tissues or cells is calculated by determining the DNA content and expressing the rate as cpm/μg DNA. The proteins of the cell and medium fractions of cultured cells are analyzed separately in order to study secretion. Morphological changes in cultured cells are recorded by polaroid photomicroscopy.

B. The level of proline hydroxylation in collagen of cells or tissues is measured by a dual labeled proline method which was devised in this laboratory and eliminates the necessity for hydrolysis. To measure the level of lysine hydroxylation, cells or tissues are labeled with radioactive lysine, and hydrolyzed collagenase digests are chromatographed on Dowex-50 in 2N HCl, which separates lysine and hydroxylysine.

C. Prolyl and lysyl hydroxylases are measured by $^{3}H_2O$ release from ^{3}H-proline or ^{3}H-lysine labeled unhydroxylated collagen prepared by incubating chick embryo frontal bones with the labeled amino acid in the presence of the iron chelator α, α-dipyridyl, which inhibits hydroxylation.

D. Collagen types synthesized in cultured cells or tissues are determined by analysis of ^{14}C-or ^{3}H-proline labeled, denatured collagen using electrophoresis in sodium dodecyl sulfate-polyacrylamide slab gels. Disulfide bonds are detected by running samples with and without dithiothreitol and observing alterations in the positions of a chains. Radioactive proteins are detected by fluorography.

E. Cyclic 3', 5' adenosine monophosphate(cAMP) is measured by a competitive binding assay. Cells are extracted with trichloroacetic acid, the cAMP purified by passing the extract through a cation exchange resin column and the extent of binding to a purified cAMP-dependent protein-kinase is measured by competition against ^{3}H-cAMP.

F. Proteoglycan synthesis. Tissues or cells are incubated with $^{35}SO_4$ and then homogenized in 4 M guanidine hydrochloride to dissociate proteoglycans. After removing insoluble material by centrifugation, the supernatant solu-

tion is passed over a gel filtration column to remove free $^{35}SO_4$, the proteoglycans in the void volume fraction are collected and radioactivity measured to determine the extent of incorporation.

Cell lines used in these studies

Chick embryo fibroblasts: prepared by digesting frontal bones from 15 day chick embryos with crude collagenase and culturing the released cells. Subcultures in the second to fifth passage are used.

Chick embryo chondrocytes: prepared from sternum.

L-929--a line of mouse embryo fibroblasts established about 30 years ago by use of a chemical carcinogen.

BALB-3T3--a contact inhibited line of mouse embryo fibroblasts isolated by Todaro and Aaronson and subclones of this line, P3 and 714, which are more stringently contact-inhibited than the original culture, and P13, which has lost density dependence.

Ki-3T3-234--a line established by Aaronson by transformation of BALB 3T3 cells with Kirsten sarcoma virus. These cells do not produce viral particles unless super-infected with murine leukemia virus.

Mo-3T3--BALB 3T3 cells transformed with Moloney sarcoma virus.
SV-3T3--BALB 3T3 cells transformed with SV-40 virus.

Embryonic and adult human diploid fibroblasts and primary Syrian hamster embryo(SHE) fibroblasts were purchased commercially.

NQT-SHE--A cell line derived by 4-nitroquinoline-1-oxide transformation of SHE cells.

BALB 3T3 subclones 3 and 714 were transformed with a temperature sensitive mutant of Kirsten sarcoma virus (KSV) containing Moloney leukemia virus (MLV) helper to give a mass infected and transformed line, tsKi(MLV)-3T3. Control lines infected with only MLV were also produced (MLV-3T3). A non-producer subclone, tsKi-3T3-714 was derived from the transformed culture of 3T3-714.

Major Findings:

A. The role of ascorbic acid in connective tissue metabolism.

On the basis of previous experiments, we have proposed a model in which defects in collagen and proteoglycan synthesis in scurvy are caused indirectly by ascorbate deficiency, rather than through the role of ascorbate in the biosynthetic pathways of these components. It had been concluded previously that the failure to accumulate collagen in scurvy was due to defective proline hydroxylation or procollagen secretion. Tissue ascorbate levels decrease rapidly after guinea pigs are placed on a vitamin C-deficient diet and after two weeks the animals become anorexic. This leads to a

chronic fasting state with associated weight loss and the animals die after four weeks. The rates of collagen and proteoglycan synthesis are coordinately decreased during the last two weeks and the decreases are correlated with the rate of weight loss. In our model, the major cause of decreased synthesis of the extracellular matrix components is the change in the concentrations of serum hormones during the fasting state of scurvy. In several systems, fasting has been shown to induce changes in circulating hormones or growth factors, many of which are known to affect proteoglycan and/or collagen synthesis. For example, glucocorticoids inhibit collagen synthesis and their levels in serum increase upon fasting. In the rat, there was a decrease in serum somatomedin C/IGF I levels which paralleled a decrease in [^{35}S]sulfate incorporation induced by fasting.

We have tested this model using two criteria. The first required that there should be changes in hormone levels in scorbutic sera similar to those observed in fasted animals. The second criteria required that the scorbutic defects in extracellular matrix component synthesis observed in vivo could be duplicated in a cell culture system in which cells were cultured in scorbutic guinea pig serum supplemented with vitamin C. This would prove that the defect was induced by changes in a humoral factor and was not related to a direct role of the vitamin in the biosynthetic pathway of either component.

1. Hormone assays.

Sera samples from control guinea pigs, those on a vitamin C deficent diet for various times, and those acutely fasted but supplemented with ascorbate for 4 days, were assayed for cortisol and T3 by radioimmune assays (Hazleton Labs). Somatomedin C/IGF I was measured by a mitogenic assay using BALB 3T3 cells, which require PDGF, EGF, and IGF I for growth. EGF was measured by a radioreceptor/competition assay.

Cortisol levels increased from approximately 60-70 µg/dl in control sera to 225-258 µg/dl in scorbutic sera. The increase occurred only during the third and fourth weeks of scurvy, which coincides with the period of weight loss. The increase was similar to that in fasted sera where the cortisol levels rose to 162-215 µg/dl.

There were no significant changes in the levels of T3. There was a loss of mitogenic activity from scorbutic sera, but only after the guinea pigs had been on the vitamin C deficient diet for two weeks, which correlates with the time when weight loss commenced. Sera from acutely fasted animals showed a similar loss of mitogenic activity. Mitogenic acitivity could be restored to scorbutic guinea pig sera by the addition of IGF I. This result coupled with the finding that there was no difference in the level of EGF in normal and scorbutic sera indicated that there was a decreased level of IGF I/ somatomedin C in the scorbutic sera.

2. Duplication of the scorbutic defect in extracellular matrix component synthesis in chondrocyte cultures in the presence of ascorbate.

Chick embryo sternal cartilage chondrocytes were prepared and cultured in

medium containing serum from either normal or severely scorbutic guinea pigs for several days. Ascorbate, at concentrations saturating for proline hydroxylation and collagen secretion was added to both cultures daily. In the cells cultured in scorbutic serum, collagen and proteoglycan syntheses were decreased to 40-50% of the values for cells cultured in normal guinea pig serum. Similar decreases were observed when fasted guinea pig serum was used. The level of proline hydroxylation in the collagen synthesized by cells in scorbutic serum was normal, as might be expected since ascorbate was added to the cultures.

The results of the two studies strongly support our model. They show that there are changes in hormones and growth factors in the serum of scorbutic guinea pigs during the period when the animals are in a fasting state. The exposure of normal cartilage cells to such serum results in decreased synthesis of extracellular matrix components, presumbably because of changes in the level of humoral factors, since ascorbate was available. We conclude that the growth promoting activity of vitamin C in vivo may be mediated initially through reactions other than those directly involved in collagen metabolism. These reactions would ensure sufficient food intake so as to maintain appropriate levels of circulating hormones that stimulate extracellular matrix synthesis. Failure to maintain normal levels of these hormones is probably the major factor involved in impeding growth of the skeletal system in scurvy.

3. An alternate reducing cofactor for prolyl hydroxylase.

Previous studies in this laboratory showed that microsomes from L-929 cells contain a reductant which can replace ascorbate as a cofactor for prolyl hydroxylase. The cofactor was extracted with Triton X-100 and exhibited high and low molecular weight forms on S-300 gel columns. Refiltration or trypsin treatment of high molecular weight cofactor produced additional low molecular weight form. The low molecular weight form was purified by P-2 gel filtration, and Dowex-1 and thin layer chromatography. It is ninhydrin reactive, exhibits reduced and oxidized forms with molecular weights of 240 and 460,respectively, and yielded cystine upon acid hydrolysis. The results suggest that it is a dipeptide, cysteinyl-cysteine, derived from a microsomal protein which is the high molecular weight cofactor.

No further work on this project is contemplated.

B. Production of New Collagen Types by the Chemically Transformed Cells, NQT-SHE.

1. Further characterization of the N-collagens [altered proα2(I) chains]

The collagen phenotype of a 4-nitroquinoline-1-oxide transformed line of Syrian hamster embryo (SHE) fibroblasts, NQT-SHE, was markedly altered from that of normal SHE cells, which synthesized mainly type I procollagen $[\text{pro}\alpha 1(I)]_2$ $\text{pro}\alpha_2(I)$. Total collagen synthesis in the transformant

was reduced to about 30% of the control level primarily because synthesis of one of the two subunits of type I procollagen, proα1(I), was completely suppressed. The major collagenous products synthesized consisted of two polypeptides, designated as N-33 and N-50, which could be completely separated by precipitation with ammonium sulfate at 33% and 50% saturation, respectively. N-33 migrated similarly to proα2(I) on SDS polyacrylamide gel electrophoresis and N-50 migrated slightly more slowly. Their helical forms, like the α2(I) helix, were unstable as evidenced by their sensitivity to pepsin, but α-chains could be obtained if digestion was carried out at 4°C with high ratios of protein:pepsin. Peptide maps of N-33α and N-50α chains generated by Staphylococcus V8 protease and cyanogen bromide and analyzed on SDS gels indicated a homology with α2(I) chains. Nevertheless, the migration of two of the V8-derived peptides differed slightly in all three maps and a doublet was observed for a cyanogen bromide peptide derived from N-33α and N-50α that corresponded to α2 CB4. These results suggest that the N-collagens are altered proα2(I) chains and that they differ from each other. Considering their similarity to proα2(I), it was surprising to find that the N-collagens were secreted to the same extent as was type I procollagen from SHE cells, and that there were no disulfide bonds between N-collagen chains. Intrachain disulfides were present. It seems likely that transformation by 4-nitroquinoline-1-oxide induced one or more mutations in the proα2(I) structural gene while suppression of synthesis of the proα(I) subunit may be due to a mutation in the regulatory region of its gene or in a general regulatory gene.

2. Investigaton of the role of proline hydroxylation and helical structure in the secretion of N-collagens

Kinetics of secretion of hydroxylated and unhydroxylated type I procollagen from SHE cells and of the altered proα2(I) chains from the transformed SHE cells were studied. Hydroxylation was prevented by omitting ascorbate from cultures or adding α,α'-dipyridyl. As expected, the rate of secretion of type I procollagen from the normal cells was inhibited when proline hydroxylation was inhibited. In contrast, the rate of secretion of altered proα2 chains from NQT-SHE cells was not affected by inhibition of hydroxylation and the rate was equivalent to that for hydroxylated type I secretion in the normal cells. These results are unexpected since previous studies have established that the helical form of procollagen involving three subunits appears to be required for optimal secretion. Therefore procollagens which are nonhelical at 37°, should not be optimally secreted. The altered proα2 chains produced by NQT-SHE cells, however, are not helical at 37° even when hydroxylated, as determined by measuring melting temperatures, yet they were secreted at the normal rate. These nonhelical chains are secreted through the normal RER, Golgi pathway as determined using inhibitors which block secretion at defined points in this pathway.

Proposed Course of Research:

A. The Role of Ascorbate in Connective Tissue Metabolism

1. Further characterization of the suppression of collagen and proteoglycan synthesis in chondrocytes cultured in sera from scorbutic or acutely fasted guinea pigs.

a. General properties of the system.

The optimal concentration of normal guinea pig serum required to maintain maximal production of the extracellular matrix components will be determined. Once this concentration has been established, chondrocytes will be cultured in medium containing equivalent amounts of normal and scorbutic or fasted sera in an attempt to determine if the latter contain an inhibitor of matrix component production or lack a factor normally required to maintain maximal production.

We will assess whether decreased matrix production represents only a quantitative change or whether dedifferentiation has occurred. Fully differentiated chondrocytes synthesize type II collagen, which is tissue specific, and cartilage proteoglycan, a large molecule containing mainly chondroitin sulfate chains bound to a core protein. Analysis of the types of collagen and proteoglycan produced in cells exposed to either normal or scorbutic serum will be carried out.

b. Mechanism for decreased matrix production.

The major objective in this aspect of the project will be to ascertain whether the chondrocyte culture system is precisely duplicating the in vivo effects of scurvy and fasting on matrix production with respect to mechanism.

In calvaria and cartilage of scorbutic guinea pigs, the decreased incorporation of labeled precursor into collagen was due to a decrease in synthesis, rather than to increased degradation. Pulse-chase experiments will be carried out to determine whether the same is true in chondrocyte cultures and similar analyses will be performed for proteoglycan production.

Type II collagen mRNA levels will be measured by hybridization and cell-free translation of RNA extracted from chondrocytes cultures exposed to the different test media. If the cell system duplicates the in vivo situation, then we would expect to observe decreased mRNA levels.

In the in vivo system, the average size distribution of cartilage proteoglycan was decreased in scorbutic tissue and we will determine if this occurs in the culture system. Proteoglycans will be extracted with guanidine hydrochoride and analyzed by gel filtration on a Sepharose CL 2B column.

c. Identification of factors involved in regulation of matrix components.

The approach to be used will depend on whether the serum mixing experiments demonstrate that decreased production of components results from induction of an inhibitor or suppression of a stimulatory factor. In the case of inhibition, we would fractionate serum from scorbutic or fasted guinea pigs, while in the case of loss of a stimulatory factor, we would fractionate normal serum. In the first case, we will use chondrocytes in normal serum to test for inhibition of collagen and proteoglycan production, while in the second case we would do the reverse.

In parallel with the above experiments, we will also test factors such as cortisol and IGF I, which we have shown are modulated in scurvy and fasting, as well as other growth factors.

B. Altered Proα2(I) Chains Produced by Chemically Transformed SHE Fibroblasts

1. Sites of alterations in proα2(I) chains.

Our previous results indicated that each of the N-collagens (N-33 and N-50) contained a modification which was reflected in a peptide released by V8 protease. We will continue analyses of peptide maps from radioactively labeled α-chains on SDS/PAGE to further localize these changes. Since the regions of the α2(I) chain from which cyanogen bromide (CNBR) peptides are derived are known, we will digest CNBR peptides with V8 protease in order to determine the region from which the altered V8 peptides are derived. Preliminary experiments suggest that this is feasible. Providing that we can prepare large quantities of the purified CNBR/V8 peptide containing the alteration, we will have the peptide sequenced to determine the nature of the modification.

Alternatively, or in parallel, we will determine whether the defect can be detected at the gene level. The peptide mapping results should tell us which region of the chain is involved and this will enable us to use restriction enzymes which would cleave the DNA in that region into small fragments. This approach has been used successfully to detect a deletion in the proα2(I) gene in a patient with osteogenesis imperfecta. The choice of enzymes will be based on the structure of the proα2(I) gene which has been determined for several species. It is possible, however, that the Syrian hamster gene is sufficiently different to require more extensive investigation of the appropriate enzymes. We will carry out Southern hybridization using the appropriate DNA probe which would hybridize with the region in question. We have obtained several proα2 probes and others are available from various investigators. If there is a significant change in the sequence, such as a deletion or insertion, we should be able to detect it. If no differences are detected, other approaches will be attempted. If a modified fragment is detected, it will be sequenced.

2. Mechanism for Suppression of Synthesis of Proα1(I) Chains

Southern blotting also will be use to determine whether there has been any detectable change in the proα1(I) gene in NQT-SHE cells, since there is no expression of this gene at either the mRNA or protein level.

3. Secretory Properties of NQT-SHE Cells

It has been shown by others that proline analogs inhibit the secretion of several different collagen types, presumably because they are incorporated into the collagen chain and destabilize the triple helix. If our conclusion that the N-collagens are non-helical at 37° is correct, then the proline analogs should not affect their secretion. On the other hand, secretion of type I procollagen from the parent cells should be inhibited. These experiments are now in progress.

Significance to Biomedical Research:

Our studies suggest an entirely new concept of how vitamin C affects the regulation of connective tissue components. In addition, they indicate that vitamin C may have biological functions not previously recognized, such as the regulation of appetite. These findings should open up new avenues of research on the role of the vitamin and lead to a better understanding of its requirements for optimal growth and maintainence of health.

Experiments on the alteration of the collagen phenotype by chemical transformation should provide information on the mechanism of carcinogenesis and how gene regulation is altered as a result of transformation. These studies also will provide an insight into the structural requirements for secretion of procollagens, in particular the relationship of the helical form to secretion.

Publications:

Chauhan, U., Assad, R., and Peterkofsky, B.: Cysteinyl-cysteine and the microsomal protein from which it is derived act as reducing cofactor for prolyl hydroxylase. Biochem. Biophys. Res. Commun. 131: 277-283, 1985.

Peterkofsky, B., Majmudar, G., Bateman, J., and Prather, W.: Expression of two unusual collagens in chemically transformed hamster cells. In Fleischmajer, R., Olsen, B.R., and Kuhn, K. (Eds.): Biology, Chemistry, and Pathology of Collagen, New York, Annals of the N.Y. Acad. Sci. Vol. 46, 1985, pp. 489-491.

Spanheimer, R.G., Bird, T.A., and Peterkofsky, B.: Regulation of collagen synthesis and mRNA levels in articular cartilage of scorbutic guinea pigs. Arch. Biochem. Biophys. 246: 33-41, 1986.

Bird, T.A., Spanheimer, R.G., and Peterkofsky, B.: Coordinate regulation of collagen and proteoglycan synthesis in costal cartilage of scorbutic and acutely fasted, vitamin C-supplemented guinea pigs. Arch. Biochem. Biophys. 246: 42-51, 1986.

Setoyama, C., Hatamoshi, A., Peterkofsky, B., Prather, W., and deCrombrugghe, B.: V-Fos stimulates expression of the α_1(III) collagen gene in NIH3T3 cells. Biochem. Biophys. Res. Commun. 136: 1042-1048, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05202-19 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Isolation, Fractionation and Characterization of Native Nucleoproteins

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

O. Wesley McBride	Chief, Cellular Regulation Section	LB	NCI
R. Balachandran	Visiting Associate	LB	NCI
W. Evans	Research Chemist	LB	NCI
A. Bale	Senior Staff Fellow	CEB	NCI
A. Roux	Guest Researcher	LB	NCI

COOPERATING UNITS *(if any)* Drs. J. Minna, J. Battey and E.A. Sausville, DCT, NCI; Drs. D. Nebert and A. Jaiswal, DP, CH; Dr. E. Max, LIG, NIAID; Dr. F. Gonzalez, LMC, NCI; Dr. P. Qasba, DCBD, NCI; Dr. M. Bustin, LMC, NCI; Dr. M. Smulson, Georgetown Univ. Medical School; Dr. S. Detera-Wadleigh, NIMH

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:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standerd 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. These hybrids have been used recently to chromosomally localize α and β DNA polymerases, several P450 cytochrome oxidase genes, a gene for gastrin releasing peptide (GRP), N-Acetyl-glucosaminide β 1-4 galactosyltransferase gene, poly (ADP Ribose) synthetase genes, creatine kinase B gene (CKBB), and a low copy number sequence flanking satellite DNA. Mapping of several other genes and multigene families is in progress. Several of these probes were also found to identify restriction fragment length polymorphisms (RFLPs) which will be useful for genetic linkage studies in families and for analyzing DNAs from patients with DNA repair defects for the involvement of β-polymerase in these defects. Establishment of genetic linkage maps of human chromosomes 1 and 15 by RFLP analysis of families is also underway. In a second area of investigation, a transforming gene has geen isolated from guinea pig leukemia and identified as an altered N-ras gene. Molecular cloning and analysis of the normal and transforming guinea pig N-ras genes is in progress.

GPO 914-916

Objective:

1) Human chromosomal mapping of protooncogenes and genes involved in DNA synthesis, carcinogen metabolism, and regulation of cell proliferation and gene expression, and 2) understanding role of these genes in human neoplasia.

Methods Employed:

Somatic cell fusion and isolation and characterization of resultant interspecific hybrid cells, metaphase chromosome- and DNA-mediated gene transfer, cytogenetic analyses and tissue culture procedures, and recombinant DNA techniques.

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. Genes also can be regionally localized on a chromosome by analysis of hybrids containing well-characterized human chromosome translocations or spontaneous breaks. A large series of independent human-rodent hybrid cell lines has been isolated, subcloned to obtain relatively homogeneous segregant hybrid cell populations retaining a few specific human chromosomes, and characterized by isoenzyme analyses and karyotyping. DNA was simultaneously isolated from each hybrid cell population for digestion with restriction endonucleases, size fractionation by gel electrophoresis, Southern transfer to nylon membranes, and hybridization with various labeled, cloned DNA probes. This technique permits the chromosomal, and subchromosomal, localization of genes and comparison with non-random chromosomal aberrations in human neoplasms. It also allows detection of pseudogenes and multigene families, determination of gene copy number, identification of restriction fragment length polymorphisms (RFLPS) detected by probes at these sites, and detection of cloned chromosomal breakpoints in neoplasms. Our previous collaborative studies with several groups have resulted in the human chromosomal mapping of the p53 cellular tumor antigen gene, ten proto-oncogenes, kappa and lambda light chain and heavy chain immunoglobulin genes and pseudogenes, the α, β, and γ fibrinogen genes, calcitonin, collagen type III gene, J protein gene and pseudogenes, P_1450 cytochrome oxidase, the metallothionein multigene family on five chromosomes, a Diffuse Lymphoma transforming gene, and a chromosomal breakpoint (BCL2) in Follicular Lymphoma.

1. Mapping of human DNA polymerases β and α

Sam Wilson and colleagues have cloned genes for α and β DNA polymerases from a rat cDNA library in a λgt 11 expression vector using immunological screening. They have also subsequently cloned these genes from human cDNA libraries. Since these genes are important in cell proliferation, DNA repair, and potentially in neoplasia, an extensive series of collaborative studies were undertaken to chromosomally map these genes. Southern analyses of somatic hybrid cell DNAs with three different β polymerase

probes consisting of 5' flanking and coding sequences, contiguous middle and 3' coding sequences, and 3' untranslated sequences all indicated that the β-Pol gene is located on human chromosome 8. Examination of hybrids containing specific translocations indicate that it is located on the proximal long arm or short arm of this chromosome (8pter-8q22). Only a single copy sequence could be detected which is very large (i.e. 757bp 5' end of 1197bp cDNA is spread over at least 20-25kb of genomic DNA). A restriction fragment length polymorphism (RELP) was detected in normal individuals and tumor cell DNAs with the 5' probe; no RFLPs were detected with middle coding or 3' non-coding probes. DNAs isolated from individuals with various DNA repair deficiencies are now being analyzed with the 5' probe in an effort to detect involvement of the β-polymerase gene. Recent studies in other laboratories based upon immunochemical analyses of somatic cell hybrids indicate that the α-polymerase gene is probably located on the X chromosomal short arm. Examination of our hybrid cell panel with a 1 kb 3' coding and flanking sequence probe from a putative human α polymerase cDNA clone indicates that it is probably located on human chromosome Xp, as expected, but definitive studies are in progress to confirm this result. These studies are complicated by the detection of many hybridizing bands (probably representing a multigene family) with this probe under high stringency hybridization conditions,and the difficulty in resolving many of these sequences from highly conserved, homologous rodent sequences in the interspecific hybrid cells DNAs.

2. Chromosomal localization of human P450 cytochrome oxidase genes

Cytochromes P450 are enzymes involved in the oxygenative metabolism of endogenous and foreign substrates including almost all drugs, chemical carcinogens, and other environmental pollutants. The P450 gene family is known to comprise at least 8 classes, or clusters, of genes. The dioxin-inducible class is composed of two genes, P_1450 and P_3450, which have differing substrate preferences. Dan Nebert and colleagues have cloned both of these genes as full length human cDNAs which have been sequenced. We have previously mapped the P_1450 gene to human chromosome 15 in collaboration with Nebert et al. The P_3450 cDNA has now been used as a probe for chromosomal mapping by Southern analysis of hybrid cell DNAS. The P_1450 and P_3450 cDNAs have two broad conserved (90% and 82%) regions of homology each extending over 500 bps. Hence, the full length P_3450 cDNA probe detects both genes even under highly stringent hybridization conditions. The two genes were shown to segregate concordantly and they were unambiguously assigned to human chromosome 15. F. Gonzalez and Nebert have recently cloned genes from two different classes of P450, namely a debrisoquine inducible P450 (P450 Dbr) and an ethanol inducible P450 (P450j). In collaboration with Gonzalez and Nebert, each of these full length P450 human cDNAs have been used as probes for chromosomal mapping. The P450Dbr gene maps to human chromosome 22 and this assignment is being confirmed by examination of additional hybrid cell DNAS. The P450j probe contains human repetitive sequences (probably Alu) and requires subcloning of a unique sequence element as a probe for chromosomal mapping studies.

3. Mapping of human gastrin releasing peptide (GRP) gene

Gastrin releasing peptide, a 27 amino acid neuropeptide, is a mammalian homologue of amphibian bombesin. GRP is both synthesized and secreted by some small cell lung cancer (SCLC) lines and can also stimulate cell growth, indicating that this neuropeptide may function as an autocrine growth factor that contributes to the transformed growth properties of SCLC. The gene has recently been cloned from cDNA libraries in two laboratories. In collaboration with J. Battey et al., the gene has been chromosomally mapped to human chromosome 18 by Southern analysis of hybrid cell DNAs with a human GRP 0.9kb near full length cDNA fragment. Further analysis of hybrid cells containing breaks in chromosome 18 demonstrated that the GRP gene is present on the long arm of this chromosome distal to BCL2 and a lambda immunoglobulin pseudogene ($\lambda\psi 1$) and proximal to a metallothionein pseudogene and peptidase A. This provided a regional localization to 18q21-q23. Subsequent in situ hybridization studies by I. Kirsch confirms and further refines this localization to 18q21. It is significant that band 18q21 is the position of a common translocaton in B-cell lymphomas [t(14q32;18q21)] which is correlated with expression of a 4.0 kb and 6.5kb mRNA from an adjacent gene, BCL-2.

4. Chromosomal mapping of N-Acetylglucosaminide β1-4 galactosyltransferase gene

Galactosyltransferases constitute a family of enzymes each member of which transfers galactose from UDP-galactose to a specific acceptor molecule, generating a specific galactose-acceptor linkage. The N-acetylglucosaminide β1-4 galactosyltransferase gene was recently cloned by P. Qasba et al. from a bovine cDNA library. The identity was confirmed by several methods including sequencing and comparing the predicted amino acid sequence with that of several peptide sequences from the purified enzyme. Because of the importance of the transferases on whose selective activities the identity of each type of cell and its distinct interactions depend, it was important to chromosomally map the gene. Since the cDNA contains a long (2.7 kb) 3'untranslated region that includes two copies of Alu-equivalent sequences, it was necessary to prepare probes from shorter fragments of coding sequence. It was also necessary to reduce the stringency of hybridization to adequately detect the gene in hybrid cell DNAs with the heterologous bovine cDNA probes. Examination of the hybrid cell DNAs with the bovine probes strongly indicates that the human homologue is located on chromosome 9. Confirmation of the results and possible regional localization of the gene on chromosome 9 is in progress by analyzing the same hybrid cell DNAs with cloned probes located on 9q (c-abl) and 9p (leukocyte interferon gene family).

5. Localizalization of poly (ADP Ribose) synthetase genes

Poly (ADP Ribose) synthetase catalyzes the post-translational modification of a large number of nuclear proteins. There is recent evidence suggesting that poly (ADP-ribosyl)ation reactions may be related to many important biological events such as DNA repair, the cell cycle, cellular

differentiation, and oncogenesis. A probe for this gene has recently been cloned by Mark Smulson et al. from a human cDNA library in a λgt11 vector. The cloned insert consists predominantly of 3' non-coding sequence, and studies are in progress to confirm its identity. In collaboration with Smulson, a 1.2kb cDNA insert fragment was labeled and used as a probe to screen hybrid cell DNAs under high stringency conditions. At least three strongly hybridizing bands were detected in human DNA after digestion with six different restriction endonucleases. Preliminary experiments indicated that all hybridizing human sequences could be resolved from homologous rodent sequences in EcoRI digests. In addition, human poly (ADP ribose) synthetase sequences could be distinguished from all mouse or hamster homologues in Hind III or Bam H1 digests, respectively. Appropriate Southern blots of the hybrid cell DNAs were examined with the probe. It was found that the human sequences detected by the probe were located on three different human chromosomes. In EcoRI digests, an 8kb hybridizing sequence could be assigned to chromosome 1, and it probably is located on the long arm (1q) based upon examination of several hybrids containing breaks in this chromosome. A 5kb hybridizing sequence was assigned to chromosome 13 and a 6.5kb hybridizing doublet is located on chromosome 14 or 15. These results were unanticipated since there is no evidence for multiple functional poly (ADP ribose) synthetase genes. Cloning of these genes will probably be required to determine whether the different genes are functionally active or whether they represent pseudogenes. Probing normal lymphocyte DNAs also revealed that the probe detects restriction fragment length polymorphisms (RFLPs) with several different restriction endonucleases. It has not been determined whether the RFLPS are present on one or all three of the different chromosomes.

6. Mapping of human CKBB (creatine kinase, brain type) gene

Mammalian cells contain three nuclear genes for creatine kinase (CK) including a mitochondrial form of this enzyme. The other two creatine kinases are dimeric enzymes and exhibit a high level of amino acid homology. Heterodimers of these molecules are found in some tissues and they are enzymatically active. There is a relatively high degree of tissue specific regulation of the expression of these two genes and there is predominant expression of a single, and opposite, type in brain (CKB) and muscle (CKM). We have previously reported that expression of the CKB isoenzyme correlated with the presence of a single chromosome band (14q32) in somatic cell hybrids. The CKM gene was recently cloned in another laboratory and mapped to human chromosome 19. Sausville and Kaye have recently cloned the CKB gene from a human cDNA library and we have undertaken chromosomal mapping of the gene in collaboration with them. This has significance because the gene is expressed at extremely high levels in small cell lung carcinoma (SCLC) cells. It is also of interest because a probe for non-coding portions of the gene detects two hybridizing EcoRI fragments in human DNA whereas only one of these fragments can be explained by the restriction pattern of the gene cloned from genomic DNA. Southern analysis of somatic cell hybrid DNAs with a 100bp probe derived entirely from the cDNA 3' noncoding sequence shows that the

two hybridizing bands are clearly located on different chromosomes, and chromosomal assignments are in progress. Two alleles of the larger hybridizing EcoRI fragment are also detected at nearly equal frequencies in DNAs isolated from ten different normal individuals indicating that the probe will be valuable in RFLP analyses.

7. Chromosomal location of low copy number sequence (LCNS) flanking human satellite DNA

A low copy number sequence (LCNS) in close proximity to satellite DNA was cloned from the African green monkey genome by A. Maresca, R. Thayer, and M. Singer (as described in their report). This sequence is highly conserved, and closely flanks satellite sequences, in both human and mouse species. To evaluate the human chromosomal location and copy number, human-rodent somatic cell hybrid DNAs were screened with two different probes prepared from the monkey LCNS flanking satellite DNA. Both probes mapped to human chromosome 4. The simple hybridization pattern of genomic DNA restriction fragments (i.e. single band in most cases) with LCNS probes and the localization of hybridizing sequences to a single human chromosome both suggest that the LCNS probe identifies a single copy sequence in the human genome. Hybridization of the LCNS probe with a partial Taq I digest of human genomic DNA revealed a 750-800bp hybridizing sequence and a hybridizing "ladder" of fragments with a 800-900 bp spacing starting at a size of 2.7kb. This result strongly suggests that the unique sequence is flanked (within a region of 2kb) by a tandemly repeated sequence which is most likely a human satellite DNA sequence. The LCNS probe was also found to identify a polymorphic Pst I site in human DNAs.

8. Mapping of human calmodulin gene and non-histone chromosonal HMG genes

A calmodulin gene has recently been cloned from a rat cDNA library in λgt11 by S. Detera-Wadleigh and we have undertaken human chromosomal mapping of the gene in collaboration with her. Somatic cell hybrid DNAs were screened with a 0.9kb rat cDNA insert probe containing 222bp of 5' coding sequence and the flanking 5' noncoding region. Multiple hybridizing bands were detected in human and rodent DNAs digested with any of six different restriction endonucleases. Analysis of EcoRI digests of somatic cell hybrid DNAs shows that the hybridizing human sequences are located on a minimum of three different human chromosomes. Human chromosomal mapping will probably require cloning the human calmodulin cDNA and using 5' or 3' non-coding sequences as a probe. This is required to exclude the possibility that the coding sequence probe is also detecting other genes which share extensive amino acid sequence homology with calmodulin within their calcium binding domains. Michael Bustin has cloned and sequenced two different HMG human cDNAs. By screening a human genomic phage library and showing a different restriction pattern for each of 125 recombinant phage examined, he has demonstrated that there must be very large number of these genes in the human genome. We have screened Southern blots of human and rodent DNAs with each of the two HMG cDNAs under highly stringent hybridization conditions. Each of the

probes identifies a very large number of discrete hybridizing fragments in human DNA and a similar pattern is observed in rodent DNAs with one probe. The background of homologous rodent sequences can barely be detected with the second probe. Using the latter probe, we are analyzing somatic cell hybrid DNAs in an effort to determine whether these sequences are all clustered on a single human chromosome or are distributed throughout the entire human genome.

B. Chromosomal Mapping of Genetic Defect in Nevoid Basal Cell Carcinoma Syndrome

Genetic linkage maps of human chromosomes can be established by analyzing DNAs from kindreds with nucleic acid probes which detect restriction fragment length polymorphisms (RFLPs). This technique has enormous potential for chromosomally mapping, and ultimately identifying, human disease loci which are currently poorly understood. The method requires neither prior knowledge of the precise nature of the gene nor a probe for the gene.

We plan to chromosomally map the nevoid basal cell carcinoma syndrome and ultimately to identify and clone the affected gene and determine the underlying mechanism leading to tumor development. The autosomal dominant mode of inheritance of this tumor susceptibility trait strongly suggests that it is based upon a single genetic defect. The high expressivity of the trait and early age of onset are factors which make genetic mapping of this disease by RFLP analysis very feasible. Previous family studies and cytogenetic analyses suggest that the gene may be located either on the short arm of chromosome 1 (1p) or chromosome 15.

This project requires cloning and characterizing a group of DNA segments which identify RFLPs at intervals not exceeding 25 centimorgans along human chromosomes 1 and 15. The DNA sequences will be isotopically labeled by nick translation and used as probes to analyze DNAs from 40 large families (i.e. C.E.P.H. families). Standard genetic linkage computer programs will be used to analyze the results thereby permitting the construction of a complete genetic linkage map of chromosomes 1 and 15 based upon recombination frequencies between pairs of RFLP markers. DNAs from families segregating the nevoid basal cell carcinoma syndrome them will be analyzed with these same probes. Analyses of the recombination frequency between the disease locus and the various RFLP probes will permit precise localization of the defect.

1. DNA has been isolated from peripheral lymphocytes of 40 unrelated individuals to be used to detect RFLPs.

2. DNA from 10 individuals has been digested with 12 different restriction endonucleases, size fractionated by gel electrophoresis, and transferred to nylon membranes for Southern hybridization with probes. Ten sets of replicate blots are available.

3. Human chromosome 15 and chromosome 1 recombinant DNA λ phage libraries have been obtained from Los Alamos National Laboratory. A group of 125

recombinant phage have been plaque purified from the chromosome 15 library and DNA isolated from confluent plate lysates. Studies are in progress to identify those recombinants containing unique sequence human DNA inserts which identify high frequency RFLPs.

4. Interspecific somatic cell hybrids have been isolated after fusing rodent fibroblasts with human cells containing specific translocations involving chromosome 15. Somatic cell hybrids retaining only specific portions of chromosome 15 are being characterized and will permit localization of each RFLP probe to a specific region of this chromosome.

5. Arrangement are in progress to obtain DNAs from 40 kindreds (C.E.P.H. DNAs) to construct a genetic linkage map of human chromosomes 1 and 15.

6. Families segregating the basal cell carcinoma tumor susceptibility trait are available. Blood samples have been collected from all these individuals. Peripheral blood lymphocytes from about 40 of these individuals have been immortalized by EBV transformation, expanded, and DNA isolated.

C. Identification of Transforming Genes in Guinea Pig Leukemia (GPL) and Human Chronic Myelogenous Leukemia (CML)

A Guinea Pig Leukemia model developed by W. Evans has many similarities to human CML. Identification of the transforming genes in these leukemias is required to firmly establish this relationship. Collaborative studies with W. Evans have been undertaken for the purpose of identifying and cloning these transforming genes.

1. We have identified a transforming gene in GPL cells by the standard focus forming assay after transfection of NIH 3T3 cells with GPL DNA. Injection of these transformed 3T3 cells into syngeneic or nude mice rapidly resulted in large tumors. DNA transforming activity was unaltered by complete digestion with EcoRI.

2. Southern analysis of DNA isolated from the transformed mouse cells (foci or tumors) demonstrated the presence of guinea pig repetitive DNA sequences and a guinea pig N-*ras* gene.

3. To facilitate isolation of the guinea pig N-*ras* transforming gene, a complete EcoRI digest of 5 mg transformed mouse DNA was size fractionated by Bull's Eye preparative agarose gel electrophoresis. Fractions enriched in N-*ras* were detected by analytical gel electrophoresis and Southern hybridization. N-*ras* enriched DNA has been ligated into an EMBO λ phage vector, packaged, and the altered N-*ras* is being cloned from this library. Cloning of the normal guinea pig N-*ras* is underway using the same procedures. Recombinant phage containing the altered N-*ras* and the normal homologue will each be tested for transforming activity in the NIH 3T3 cell assay.

4. Subsequent studies will include cloning the normal and transforming N-ras cDNAs, sequencing the genes to detect the putative mutation resulting in transforming activity, insertion of the genes into appropriate expression vectors, and analyzing the effect of the transforming gene inserted into normal guinea pig bone marrow cells.

Publications:

Nau, M.M., Brooke, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F., and Minna, J.D.: L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318: 69-73, 1985.

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Kant, J.A., Fornace, A.J., Jr., Saxe, D., Simon, M.I., McBride, O.W., and Crabtree, G.R.: Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication acompanied by transposition and inversion. Proc. Natl. Acad. Sci. USA 82: 2344-2348, 1985.

McBride, O.W., Merry, D., and Givol. D.: The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). Proc. Natl. Acad. Sci. USA 83: 130-134, 1986.

McBride, O.W., Zmudzka, B.Z., and Wilson, S.H.: Chromosomal location of the human gene for DNA polymerase beta. Proc. Natl. Acad. Sci. U.S.A. (in press).

Max, E.E., McBride, O.W., Morton, C., and Robinson, M.A.: The human J chain gene: chromosomal localization and associated restriction fragment length polymorphisms. Proc. Natl. Acad. Sci. U.S.A. (in press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05203-18 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 charecters 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	Fogarty Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Protein Chemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Our goal is the development of methods for the specific isolation of antigen-reactive cells (ARC) in order to study the molecular interactions that occur in their immune reactions. Populations of cells containing ARC are tested for binding to the cell surface antigens of target cells when the target cells and/or their antigens have been attached to insoluble surfaces. Separated ARC populations are tested for cytotoxic effector cells (CTL) and their precursors, and for activity in allograft rejection and graft-versus-host and mixed lymphocyte reactions. T cell subpopulations are separated and characterized by reactions with specific reagents such as monoclonal antibodies to cell surface antigens by flow cytometry. Surface molecules of target cells, in particular class I MHC antigens, are isolated to test their binding to ARC.

GPO 914-918

Project Description

Objectives:

The goal of this project is the development of methods for the specific isolation of immune cells, particularly for different types of antigen-reactive cells (ARC) involved in cellular immune reactions to alloantigens, to study the ontogeny of these cells, to study their roles, alone and in combination, in several *in vitro* and *in vivo* cellular immune reactions, and for their immunochemical characterization in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. In particular, we study the differentiation of cytotoxic effector cells (CTL) from precursors (CTLp) in thymus and spleen, the molecular reactions between immune cells and antigens bound to target cell surfaces, and the role of T cell subpopulations in *in vivo* cellular immune phenomona such as graft rejection and the graft-versus-host reaction.

Methods Employed:

Antigen-reactive cells (ARC) obtained from normal mice or from mice immunized with allogeneic tumor cells or normal cells are incubated on surfaces coated with allogeneic or syngeneic target cells or on surfaces coated with antibodies to cell surface molecules. Graft-versus-host activity (GVH) of nonadherent cells or of released adherent cells is measured by the Simonsen spleen weight assay in neonatal F1 mice. CTL activity is measured by ^{51}Cr release from target cells. Stimulation by alloantigen is measured by the mixed lymphocyte reaction and by generation of CTL *in vitro*.

T cell subpopulations from thymus and spleen are separated and characterized by their reactions with specific monoclonal antibodies to cell surface differentiation antigens. Cell surface molecules of target cells are isolated to test their binding to ARC. Monoclonal antibodies are prepared that react with CTL surface antigens.

Major Findings:

A. Class II MHC Antigen-independent Early Activation of CTL Precursors.

In collaboration with Dr. L. Romani of the University of Perugia, a possible requirement for class II major histocompatibilty complex (Ia) molecules in the initial activation of cytotoxic T lymphocyte precursors (CTLp) for allocytotoxic responses was investigated. To avoid possible interaction with other alloreactive cell types, a higly purified population of Lyt-2^+ splenocytes was used as a source of CTLp. In the light of preliminary results indicating that Lyt-2^+ CTLp, even in the presence of interleukin 2 (IL2), could best be triggered into mature CTL *in vitro* by cells known to be Ia^+, we examined whether an interaction of CTLp with Ia antigens (either on syngeneic accessory cells or on allogeneic stimulators) played a role in the development of allocytotoxicity. Results from experiments done with C57BL/6 Lyt-2^+ splenocytes co-cultured with P815 stimulator cells and IL2 showed that the early activation of CTLp was independent of Ia^+ syngeneic accessory

cells: (a) flow microfluorometry analysis of the responder population at the beginning or after 1 or 3 days of co-culture did not reveal the presence of Ia^+ cells; (b) procedures for removal of residual Ia^+ cells or of dendritic cells from the responder population before co-culture did not affect the development of cytotoxicity; (c) co-culture with monoclonal antibodies against syngeneic Ia^b antigens did not inhibit the CTLp activation. By comparing an Ia^+ P815 tumor line with its Ia^- clone as allogeneic stimulator cells, it was found that the CTLp activation was also independent of Ia alloantigen on the stimulator cells. The response against both the Ia^+ and the Ia^- stimulator cell types was not inhibited by monoclonal anti-L3T4 present in the co-culture, indicating that these responses were not affected by residual L3T4 helper cells.

B. Further Characterization of the Antibody Response to an "Xenogenized" Tumor Cell Line.

"Xenogenization" (i.e., induced mutation in tumor cells to increase their immunogenicity) is a potential approach to cancer immunotherapy of tumors that fail to elicit an effective immune response. Also in collaboration with Dr. Romani, the humoral response to a chemically xenogenized murine tumor cell line, previously identified only through its ability to elicit a cellular immune response was further characterized. Histocompatible mice were given several injections of live cells of the xenogenized tumor. Ten days after each immunization, pooled sera from different animals were analyzed for Ab content by means of flow microfluorometry analysis and CELISA assay. The results reveal that antibodies of both IgG and IgM classes capable of binding the xenogenized tumor can already be detected after one single sensitization. However, the Ab titer gradually increases through subsequent immunizations, reaching a peak level after 3-4 injections at a time when most of the humoral response is made up of antibodies of IgG class. The specificity of the anti-xenogenized tumor hyperimmune sera was subsequently investigated by its reaction with the parental, non-xenogenized line and with normal tissue cells of the same or allogeneic haplotypes. The data obtained point out that cross-reactivity with the parental line could be completely removed by absorption of the hyperimmune sera on parental cells, which removed most of the IgM antibodies. Moreover, the presence of an excess of anti-parental antibodies on the xenogenized tumor cells does not prevent the subsequent binding of the hyperimmune absorbed serum, thus indicating that the novel determinant(s) recognized on xenogenized cells are not spatially related to those shared with the original parental tumor. In addition, the hyperimmune absorbed serum does not cross-react with normal hemopoietic or lymphoid cells of the same ($H\text{-}2^d$) or allogeneic $H\text{-}2^b$ and $H\text{-}2^k$ haplotypes. Furthermore, no alien histocompatibility antigens of $H\text{-}2^b$ or $H\text{-}2^k$ haplotypes could be detected on the xenogenized tumor cell surface. Taken together, these data provide evidence that chemical xenogenization of a murine lymphoma leads to the appearance of novel determinant(s) detectable by specific antibodies.

C. Tumor Targeting by Attachment of Antigens

We are developing another potential approach to immunotherapy of tumors, related to "xenogenization". This is to immunocchemically attach antigens to tumor cells, in order to increase their susceptibility to cellular immune reactions. We are initially using Class I MHC molecules because of their strong immunogenicity for cellular immunity. Antibodies to public specificities of the Class I molecules are used to attach allogeneic affinity-purified Class I molecules to mouse lymphoid tumor cells. Using purified mouse and rat monoclonal antibodies, even in large antibody excess, we have not been able to demonstrate attachment of allogeneic Class I molecules to the cell surface, although these antibodies successfully crosslink the Class I molecules in an ELISA assay where the well is first coated with one Class I antigen. On the other hand, rabbit polyclonal anti-mouse Class I MHC antibody does successfully bind allogeneic Class I molecules to the cell surface. We are now exploring how to effectively use the monoclonal antibodies to attach Class I antigens to cells, and are studying the use of Class I antigen-coated tumor cells as immunogens and targets for cellular immunity.

Significance to Cancer Research:

T cells are thought to be directly involved in anti-tumor immunity by means of cell-mediated tumor rejection, its augmentation and suppression. Extending our knowledge of the antigen-binding characteristics and immune reactivities of different T cell populations, and of the relationship of cell-mediated and antigen responses to antigens of tumor cells, may help in designing purifications of lymphocytes reactive against tumor cells, and in specific suppression of the graft-versus-host reaction, which is a serious iatrogenic complication of cancer therapy with bone marrow transplantation.

Proposed Course of Research:

In addition to continuing the development of general cell-separation methods based on binding of Ig-coated cells to anti-Ig-coated surfaces, we plan to continue the study of the interaction of CTL with the macromolecules (such as Class I MHC antigens) involved in their binding to target cells, to study the molecular requirements for immunogenic antigen presentation to precursors of CTL, and to continue to develop systems for targeting tumor cells by attachment to them of class I MHC molecules.

Publications:

Romani, L., Puccetti, P., Fioretti, M.C., and Mage, M.G.: Humoral response against murine lymphoma cells xenogenized by drug treatment in vivo. Int. J. Cancer 36: 225-231, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05214-15 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

S. H. Wilson	Medical Officer	LB NCI
F. Cobianchi	Guest Worker	LB NCI - 8 mo.
P. Kumar	Visiting Fellow	LB NCI - 5 mo.
D. SenGupta	Visiting Fellow	LB NCI
B. Zmudzka	Visiting Associate	LB NCI
P. Kedar	Visiting Fellow	LB NCI - 5 mo.

COOPERATING UNITS *(if any)* J. Mitchell, NCI; J. Oppenheim, NCI; H.R. Guy, NCI; G. Zon, FDA; F. Robey, FDA; E. Baril, Worcester Foundation for Experimental Biology; P. Hoffee, University of Pittsburg; K. Williams, Yale University; R. Karpel, University of Maryland; J. Collins, Virginia Commonwealth University.

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Biosynthesis Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	4.5	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We continued studies of cDNAs to the mammalian DNA replication proteins, α-polymerase, β-polymerase and single-stranded nucleic acid binding protein (helix destabilizing protein). Our full-length cDNA for the binding protein was subcloned in an expression vector and the protein was over produced in E. coli and isolated in mg quantities. The amino acid sequence of this protein was identical with that of the protein deduced from the cDNA and with the sequence of the A1 protein purified from mammalian hnRNP particles. Protein-nucleic acid binding studies revealed that the C-terminal domain of the protein is critical for tight binding.

The sequence of our cDNA to rat β-polymerase was determined. Properties of the sequence 5' of the first ATG in the cDNA suggested that this ATG is the initiation codon for synthesis of the 318 amino acid protein encoded by the single long open reading frame of the mRNA. This cDNA was used to probe a cDNA library of poly A^+ RNA from a human cell line and an analogous human cDNA was isolated and sequenced. The human and rat cDNAs were 90% homologous and the proteins deduced from the two open reading frames were similar (95% matched residues).

Our rat cDNA to α-polymerase (1.2 kb) was sequenced and found to contain only a short open reading frame, presumably corresponding to the C-terminal end of the α-polymerase catalytic polypeptide. Antibody against a synthetic oligopeptide with this sequence reacted with purified α-polymerase and immunoprecipitated α-polymerase from solution. A panel of longer cDNAs was obtained from a library to human poly A^+ RNA using our antibody to the Hela α-polymerase, catalytic polypeptide.

These various human and rat cDNAs are now being used as probes to study expression of mRNAs, to identify polymorphisms in genomic DNA, and to conduct chromosomal localizations.

Project Description

Objective:

The ultimate objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells. Our main approaches are to study the enzymology of DNA enzymes and eventually to examine DNA replication in vitro using purified DNA 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 of DNA replication in the mammalian cell.

Methods Employed:

Procedures for assay and purification of DNA polymerases and other DNA proteins from mammalian cells and tissues are being used. These procedures involve subcellular fractionation, various types of ionic separation, gel filtration chromatography, immuno-binding procedures, HPLC, affinity chromatography, and assays for DNA polymerases using a variety of methods. Cell culture and fractionation procedures and characterizations of reaction products are performed using conventional techniques such as rate-zonal centrifugation, scintillation spectrophotometry, thin-layer chromatography, and gel electrophoresis. Recombinant DNA technology is being used for preparation of cDNAs, apppropriate expression vectors, and genomic DNA sequences spanning genes for the various DNA proteins under study.

Major Findings:

A. Structure-Function Relationships of a Model Polymerase, E. coli DNA Polymerase I Large Fragment (Pol I l.f.)

Termination Mechanism

The model DNA polymerase, Pol I l.f., generally forms DNA in a processive fashion, remaining associated with the template:nascent chain for many dNMP additions. The amount of termination after any given dNMP addition can be determined by analysis of reaction products on a sequencing gel, and in the past we exploited this method to study the termination mechanism using homopolymer template-primer systems. In collaboration with G. Zon, we now are studying termination using a natural DNA (ϕX174) template-primer system. The results indicate that regions of single-stranded DNA that are secondary structure-free contain termination sites for this enzyme. Synthesis is processive until a termination site is encountered, at which point the chance for termination increases. This analysis has shown that termination is higher at each position along the template where the incoming nucleotide is a pyrimidine. This phenomenom also was observed with 40 residue long synthetic oligonucleotide templates, and hence, this type of termination could be studied with selected modifications of individual template residues. Modifications altering hydrogen bonding patterns and protein phosphate

contacts were evaluated. These modifications, all of which can be formed by carcinogens, lead to increased termination. The results emphasize an "incoming nucleotide effect" in the mechanism pol I 1.f.

B. Structure and Function Relationships 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. Characterization of rat alpha polymerase cDNA:

A new polyclonal antibody against the α-polymerase catalytic polypeptide (described in last year's report) was used to screen a cDNA library of newborn rat brain polyA$^+$ RNA in λgt11. A positive phage was identified and plaque purified. This phage, designated λpolα1.2, also was found to be positive with an antibody against Drosophila α-polymerase. The insert in λpolα1.2 was subcloned into pUC9 and then into M13 for sequence analysis. The insert (1.2 kb) contained a poly A sequence at the 3' terminus and a short in-phase open reading frame at the 5' terminus. In collaboration with F. Robey a synthetic oligopeptide (8 amino acids) corresponding to the open reading frame was used to raise antiserum in rabbits. Antibody affinity purified from this serum was immunoreactive against purified α-polymerase by ELISA and was capable of immunoprecipitating α-polymerase. This indicated the λpolα1.2 insert encoded an α-polymerase epitope and suggested that the cDNA corresponded to an α-polymerase mRNA. This was confirmed in hybrid selection experiments using pUC9 containing the cDNA insert and polyA$^+$ RNA from newborn rat brain; the insert hybridized to mRNA capable of encoding α-polymerase catalytic polypeptides. Southern blot analysis of rat genomic DNA was consistent with a low copy gene. Northern blot analysis of newborn rat brain poly A$^+$ using the nick translated insert as probe revealed a low abundance hybridizing RNA species of approximately 5200 bases.

2. Identification and characterization of human alpha polymerase cDNAs:

A λgt11 cDNA library of polyA$^+$ RNA from a human teratocarcinoma cell line was screened with our antibody to the HeLa 183 KDa alpha polymerase catalytic polypeptide. Eight positive phage were identified and plaque purified. The cDNA insert of one positive clone, λpolα42, was selected for detailed study. This insert (2400 bp) was used to probe Northern blots of various mammalian polyA$^+$ RNAs and was found to be hybridized with a species of about 4,000 bases. This cDNA did not cross hybridize with the rat alpha polymerase cDNA described above, probably because the rat cDNA represented the 3' untranslated region of the mRNA. λpolα42 also produced an epitope for a second independently derived antibody to α-polymerase.

3. Attempts to isolate rat cell lines with amplified α-polymerase genes:

In collaboration with P. Hoffee, we have isolated a panel of rat hepatoma cell lines that are resistant to the α-polymerase inhibitor butylphenyldeoxyguanosine. This inhibitor competes with dNTP for binding sites on the enzyme. It was expected that resistant cell lines could be nucleotide pool size mutants or permeability mutants, as well as various types α-polymerase mutants. Levels of α-polymerase enzyme activity were found to be ~5-fold higher in two of the resistant cell lines than in the parent cells. These drug resistant cell lines also were found to contain higher amounts of α-polymerase catalytic subunit as revealed by Western blotting experiments. Genomic DNA from these cell lines is being tested with probes based upon the rat and human α-polymerase cDNA probes described above. These findings are still in the preliminary stage and will be described in more detail in a later report.

4. Characterization of human beta polymerase cDNA:

A cDNA library from polyA$^+$ RNA of a human teratocarcinoma cell line in phage λgt11 was screened with a fragment of the rat β-polymerase cDNA described in last year's report. Five positive phage were identified and plaque purified. The cDNA of one positive clone, λpolβ-h2, selected for detailed study was 1257 bp. This insert was sequenced and found to contain the coding region for β-polymerase, as well as 163 bp and 137 bp from the 5' and 3' untranslated regions, respectively. The primary structure of human β-polymerase (318 amino acids, M_r=36,133) deduced from the cDNA was similar to rat β-polymerase (95% matched residues). The greatest difference between the sequences of the human and rat cDNAs was in the 3' untranslated regions (64% matched base residues).

Most of the nucleotide differences in the coding region of the two cDNAs did not lead to an amino acid codon difference, and in the 16 cases where a codon difference occurred, 9 lead to an amino acid of similar chemical properties. The number of nucleotide differences (92) between the 954 residue coding regions permits a calculation of the substitution rate, assuming the sequences diverged 80×10^6 years ago. An overall substitution rate of ~1 per site per 10^9 year was obtained. This value is quite low and is about one-half that of other mammalian coding regions characteristic of low overall substitution rates (HPRT, ACTH, Histone H2A, and parathyroid hormone). The ratio of nonconservative to overall nucleotide differences (0.24) suggested that β-polymerase is subject to fairly strong selective pressure, though not as strong as that for α-actin, histones, or helix-destabilizing protein. Plots of computer-derived secondary structure predictions for human β-polymerase were similar to those for rat β-polymerase and indicated 7 regions of α-helix spaced throughout the sequence and several regions of β-sheet.

Since the human cDNA appears to be a full-length copy for the protein coding region, it should be possible to produce large amounts of active native enzyme in *E. coli* through use of appropriate expression vector

systems. This would greatly facilitate detailed structure-function studies of β-polymerase, a research area of particular promise since this enzyme is the simplest naturally occurring DNA polymerase known.

5. Human chromosomal localization and DNA polymorphism studies:

In collaboration with O.W. McBride, human genomic DNA was subjected to Southern blot analysis using the α-polymerase and β-polymerase cDNAs as probes. The results to date indicate that the β-polymerase gene is single copy and is located on chromosome 8. 20-25% of humans have a polymorphism in the 5' end region of the gene.

6. Survey of mRNA levels in mammalian cells using α-polymerase and β-polymerase cDNAs as probes:

Using Northern blot analysis, in collaboration with J. Mitchell, J. Collins, P. Hoffee, J. Oppenheim and others, we are studying mRNA levels in a variety of resting and stimulated cells. The results of these studies are still preliminary and will be described in a later report.

7. Studies of DNA polymease β genes in cultured cells with abnormal DNA repair:

In collaboration with J. Mitchell and O.W. McBride we are examining genomic DNA from cells of humans with inherited disorders associated with defective DNA repair. The results to date fail to indicate deletion or rearrangements of the β-polymerase gene in the abnormal human cells lines tested.

C. Studies of Helix Destabilizing Protein cDNA Clones

Our full-length cDNA to mammalian helix-destabilizing protein was resected with Bal31 nuclease and then subcloned in an E. coli vector under expression control of the λP_L promoter. E. coli grown under appropriate conditions over expressed the mammalian helix-destabilizing protein in amounts equal to about 50% of total cell protein. The protein was purified from these extracts using a new one-step procedure and then the amino acid sequence was determined. Two important findings were obtained. First, the sequence of the 320 amino acid protein deduced from the open reading frame of the cDNA matched the sequence of the manufactured protein, and second, the manufactured protein was found to be identical with the A1 protein of mammalian hnRNP particles. Classical equilibrium binding studies revealed that the protein binds tightly to singlestranded nucleic acid and that truncation of the C-terminal end leads to decreased binding affinity. Further structure-function studies of this protein are in progress.

Significance to Cancer Research:

Detailed knowledge of the mechanisms of DNA synthesis is vital to our understanding the molecular biology of neoplasia. Many approaches are being used to

investigate DNA synthesis in normal and neoplastic cells, and advances on all levels will prove useful in preventing, treating, and ultimately controlling cancer. Activity of DNA synthesizing proteins is necessary for maintenance of a rapid rate of cell division, and in some cases there is evidence that these proteins may play an important role in the development of the neoplastic state.

Proposed Course of Research:

To characterize the genomic organization of human and rat genes for the DNA proteini under study and to examine structure-function relationships of protein prepared by overexpression in E. coli.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05231-12 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Subunit Interactions in Enzyme Chemistry and Cellular Regulation

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

C.B. Klee	Chief, Macromolecular Interactions Section, LB, NCI		
M.J. Hubbard	Visiting Fellow	LB	NCI
J.L. Foster	Guest Researcher	LB	NCI
M.H. Krinks	Chemist	LB	NCI
J.R. Miller	Technician	LB	NCI
T. Jean	Visiting Fellow	LB	NCI
G.F. Draetta	Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)* Dr. J. Schiloach, NIAMMD; Dr. P. Cohen, University of Dundee, Scotland; Dr. L. Heppel, Cornell University, Ithaca, NY; Dr. P. deCamilli, University of Milan, Milan, Italy; Dr. J. Wolff, NIAMDD; Dr. A.R. Means, Baylor University of Houston, Texas; Dr. J. Haiech, Montpellier, France.

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SECTION
Macromolecular Interactions Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	5.5	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.)*

Stimulus response coupling mediated by calcium and calmodulin involves several steps. (1) The calcium release from the endoplasmic reticulum induced by inositol triphosphate which has been shown to be modulated by intracellular calcium levels. (2) The binding of calcium to calmodulin is a cooperative and selective process that is modulated by magnesium. At physiological ionic strength, and only in the presence of magnesium, a large difference is seen between the affinities of sites III and IV (0.16 and 0.24 x $10^6 M^{-1}$), and sites I and II (0.01 and 0.07 x $10^6 M^{-1}$) for calcium. The large negative cooperativity between the calcium sites located in the two halves of the calmodulin molecule, induced by magnesium, requires the integrity of the calmodulin molecule. The magnesium-induced difference in affinity of the calcium binding sites, together with the positive cooperativity, explains the stepwise conformational changes induced by calcium. (3) The interaction of calmodulin with its target proteins involves different portions of the calmodulin molecule. Calmodulin-regulated enzymes can be divided into three classes according to their abilities to bind and to be activated by calmodulin fragments: (a) enzymes which are activated by the C-terminal fragment include the Ca^{2+} ATPase, phosphorylase kinase and B. pertussis adenylate cyclase; (b) enzymes which require both halves of the molecule such as cyclic nucleotide phosphodiesterase and myosin kinase; and (c) enzymes whose interaction with calmodulin fragments is too weak to be detected by activation, such as the calmodulin-regulated protein phosphatase, calcineurin and the multiprotein kinase. Thus, different enzymes may be activated by different calmodulin conformers and the stepwise changes exhibited by calmodulin at different calcium levels can be used to regulate different metabolic pathways.

Stimulus-response coupling by cAMP are also being studied by J. Foster who has cloned the gene of the catalytic subunit of cAMP-dependent protein kinase in Drosophila melanogaster.

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 intracellular calcium levels by inositol triphosphate, and on 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.

Methods Employed:

The project involves the study of properties of proteins, purified and analyzed by the usual techniques of protein isolation, including many types of chromatography and electrophoresis. The enzymes are studied by examination of their optical, hydrodynamic, kinetic, and immunochemical properties as well as by suitable chemical measurements and modifications. The roles of these proteins in cellular regulation are studied in cultured cell lines.

A. Calmodulin and Calcium Regulation of Cellular Activity

Stimulus-response coupling mediated by Ca^{2+} involves several successive steps: (1) transitory increase in intracellular Ca^{2+} from 10^{-7} to 10^{-6} - 10^{-5}M, (2) interaction of Ca^{2+} with the Ca^{2+} receptor, calmodulin (3) interaction of the activated calmodulin.Ca^{2+} complex with the various target proteins and (4) coordinated activation of several enzymatic reactions.

We have focused our attention on four different aspects of the calcium regulation of cellular processes: (1) regulation of intracellular calcium levels; (2) interaction of calmodulin with calcium; (3) calmodulin activation of target proteins; and (4) role of calcium and cAMP in stimulus response coupling.

1. Regulation of intracellular calcium concentration.

In many cells the increase intracellular calcium needed to initiate the calmodulin-dependent stimulation of cellular processes is induced by the second messenger inositol triphosphate (IP_3). Calcium mobilizing stimuli, such as acetylcholine, vasopressin, growth factors, and antigens, upon binding to membrane receptors, are believed to stimulate phospholipase C which catalyzes the hydrolysis of phosphatidyl inositol 4,5-biphosphate to diacylglycerol and IP_3. Whereas diacylglycerol stimulates a membrane-associated phospholipid-dependent protein kinase, protein kinase C, the water soluble IP_3 induces a release of calcium from internal stores. Subcellular fractions of neuroblastoma-glioma hybrid cells NG108-15 were used to study the mechanism of IP_3-induced calcium release. Maximum release was observed with a post mitochondrial fraction enriched in the endoplasmic reticulum enzyme glucose-6-phospha-

tase and almost devoid of the mitochondrial enzyme cytochrome oxidase, confirming the identification of the endoplasmic reticulum as the site of action of IP_3. In agreement with this identification, calcium uptake by this membrane fraction exhibits a high affinity for calcium ($K_{0.5}$ $<10^{-6}$M). The rapid ($t_{1/2}$ <15 sec) release of calcium from this microsomal fraction is dependent on IP_3 concentration with a $K_{0.5} = 10^{-6}$M and is regulated by calcium concentration. It is maximal at 2×10^{-6}M free calcium and is completely inhibited at calcium concentrations above 2×10^{-5}M. The IP_3-regulated calcium release is distinct from the previously reported GTP-induced calcium efflux (also observed in NG108-15 cell extracts) which occurs optimally at high calcium concentration, is greatly stimulated by the presence of polyethylene glycol and possibly originates from a different type of calcium sequestrating vesicle. The modulation of IP_3-induced calcium release by cytoplasmic calcium levels is an important safety mechanism which prevents deleterious effects of abnormally high calcium concentrations and allows the stimulation of protein kinase C independently of the calcium stimulus (Thierry Jean).

2. Interaction of calmodulin with calcium

At physiological concentrations of K^+ (100mM), binding of calcium to calmodulin exhibits positive cooperativity, but there is no evidence for large differences in the affinities of the sites for calcium. The calcium-binding isotherms fit well to a model assuming two sets of cooperative binding sites with binding constants of 0.04 and 0.02 x 10^6M^{-1}. Calmodulin fragments 1-77 and 78-148 also exhibit positive cooperativity and binding constants of 0.1 and .2 x 10^6M^{-1}, respectively. This small difference in binding constants between the high and low affinity sites is not sufficient to explain stepwise conformational changes. It is not possible to assign the different sites to those of a particular calmodulin fragment because the affinities of the calcium sites are different in the native protein and in the isolated peptides. Although calmodulin fragments have been shown by proton NMR to have a structure similar to the one they have in the native protein, the differences in calcium-binding properties suggest that subtle differences exist between the isolated fragments and their counterparts in the parent molecule.

Under more physiological conditions, in the presence of 1mM Mg^{2+} as well as 100 mM K^+, the binding of calcium is best fitted to two sets of cooperative sites. There is now a large difference in the affinities of the two sets of calcium sites. The binding constants are .09 and .0007 x 10^6M^{-1}. This large difference in affinities, coupled with the positive cooperativity, explains the discrepancy between apparently normal calcium binding isotherms and stepwise conformational changes induced by calcium. In the presence of magnesium there is excellent agreement between the experimental Ca^{2+} binding curve to fragment 78-148 and the predicted binding curve for the high affinity sites. Thus the high affinity sites in the presence of magnesium are in domains III and IV.

The calcium-binding properties of calmodulin in the presence and absence of magnesium indicate that, at physiological concentrations, magnesium

has a specific effect on calmodulin structure and in so doing modulates its interaction with calcium. Magnesium could exert its effects simply by preferential binding at two specific calcium sites and thereby selectively reducing their affinities for calcium. However, such a large specific effect of magnesium on two of the calcium sites is not observed with calmodulin fragments. Both halves of the molecule are affected by magnesium in a similar manner; the major effect of magnesium is to stabilize the native structure. Whereas in the absence of magnesium the calcium binding properties are different from those of the same peptides in the native protein, in its presence, the peptides exhibit binding properties similar to those of calmodulin. Thus, magnesium, in addition to its inhibitory effect on calcium binding to low affinity sites, may also play a role in keeping the two halves of calmodulin independent of each other by stabilizing the long helix connecting them which has been revealed by recent X-ray crystallographic data. These results are in contrast to the known specificity of magnesium for the high affinity sites of another calcium-regulated protein, skeletal muscle troponin C. They may reflect the different functions of these two proteins (Newton and Haiech).

3. Interaction of calmodulin with target proteins.

The most intriguing and unique property of calmodulin is its ability to interact with and activate a very large number of enzymes. In collaboration with other investigators we have extended the evidence suggesting that the interaction of calmodulin with any one of these target proteins requires the integrity of one of several different portions of the calmodulin molecule.

Calmodulin fragments 1-77 and 78-148 have been useful tools with which to dissect the requirements of different target proteins for interaction with an activation by calmodulin. The activities of these peptides compared to that of calmodulin were tested in three ways: (1) calcium-dependent activation of calmodulin-regulated enzymes; (2) calcium-dependent binding of a given enzyme to the peptides coupled to Sepharose; and (3) inhibition of calmodulin stimulation of the enzyme by the peptides. All enzymes tested interact with fragment 78-148, but only two enzymes, cyclic nucleotide phosphodiesterase and phoshorylase kinase interact with fragment 1-77. Thus, some enzymes recognize only site(s) located in the carboxyl-terminal half of calmodulin; others interact with at least two sites, one on each half of calmodulin. Among the enzymes which bind to peptide 78-148, only three are activated by this fragment; phosphorylase kinase, the plasma membrane (Ca^{2+}+Mg^{2+}) ATPase; and B. Pertussis adenylate cyclase. Similarly, only one enzyme which binds to fragment 1-77 is activated (phosphorylase kinase) whereas the other (phosphodiesterase) is not. Thus, some enzymes exhibit more stringent requirements for activation than for binding. In all cases, cleavage of calmodulin in the middle of the molecule results in a two to three orders of magnitude decrease in affinity for the target enzymes suggesting that the four domain structure is critically important for interaction. A one to one, covalent complex of calmodulin with the anti-

calmodulin drug, norchlorpromazine (CAPP-calmodulin) has been used to further characterize the different interaction sites of calmodulin with its target proteins. In this complex, the drug apparently occupies either one or the other of these sites, but not both simultaneously. CAPP-calmodulin which has preserved the four domain structure has also retained its high affinity for each of the target enzymes tested. With the exception of two of the enzymes, phosphodiesterase and myosin kinase, it has also preserved its activating ability.

Thus, calmodulin-regulated enzymes can be divided into three classes according to their abilities to bind to and be activated by the calmodulin fragments and by CAPP-calmodulin: (1) enzymes like phosphorylase kinase, the ATPase and B. Pertussis adenylate cyclase bind to and are activated by CAPP-calmodulin; (2) enzymes like calcineurin and the multiprotein kinase interact with the carboxyl-fragment but the interaction may be two weak to be detected by activation or inhibition of calmodulin stimulation. In this case CAPP-calmodulin, which has preserved a high affinity for these enzymes, is an activator; (3) the last class of enzyme, which includes phosphodiesterase and myosin kinase, needs at least two sites for activation. Neither of the fragments activates these enzymes. CAPP-calmodulin binds tightly to these two enzymes but has no effect on their activity. This classification scheme is consistent with the calcium dependence of activation by calmodulin of three of the enzymes. Phosphodiesterase and myosin kinase, which require four calcium/mol of calmodulin are believed to recognize sites on each half of calmodulin; phosphorylase kinase whose activation is directly proportional to calcium binding to calmodulin does not require full occupancy of the calmodulin sites and needs to interact with only a portion of the molecule. These considerations suggest that different enzymes may indeed be activated by different conformers of calmodulin and that the stepwise changes exhibited by calmodulin at different calcium levels can be used to regulate different metabolic pathways (M.H. Krinks, D.L. Newton, G.F. Draetta, W.-C. Ni, J. Wolff, P. Cohen).

4. Regulation of calcineurin

We have studied the calcium dependent interaction of calmodulin with calcineurin, a brain protein which is a member of the calmodulin-regulated protein phosphatase family, to identify the calmodulin interaction site(s) in one of its target proteins.

Affinity selection, a technique whereby calcineurin selects from a population of randomly modified calmodulins those species with which it prefers to interact, shows that acetylation of lysines affects calmodulin so as to interfere with its ability to interact with calcineurin. Monoacetylation of any lysine of calmodulin reduces its affinity for calcineurin five to ten fold. Multiple acetylations amplify the loss of affinity; none of the modifications are incompatible with activity. The lack of selectivity of calcineurin against any particular modified lysine indicates that the loss of affinity reflects changes induced by

the removal of the charged groups and suggests an important role for electrostatic interactions in the cooperative structural transitions which calmodulin undergoes upon binding calcineurin or calcium.

A large and specific decrease in the rates of acetylation of Lys 75 and 148 of calmodulin is observed in the presence of calcineurin. The reactivity of the same residues is greatly increased in the presence of calcium alone (Giedroc et al., J. Biol. Chem. 260: 13406-13413, 1985). Lys 75, located in the central helix, and the C-terminal Lys 148 may act as sensors of the calmodulin allosteric transitions. Their reactivity changes in opposite directions in response to calcium-induced or calcineurin-induced structural changes. The reactivity of Lys 21, decreased in the presence of calcineurin but not calcium, also reflects a conformational change induced specifically by calcineurin (A. Manalan).

We prepared monoclonal antibodies to calcineurin by in vivo immunization and in vitro hybridoma procedures. A combined solid and liquid phase screening protocol allowed us to isolate a panel of monoclonal antibodies with high and low affinities. A calmodulin binding assay was then developed to investigate the effect of these antibodies on the interaction of calmodulin with calcineurin. Calcineurin was quantitatively adsorbed to nitrocellulose filters and tested for its ability to bind radiolabeled calmodulin. The slow (24 hours) binding of calmodulin to immobilized calcineurin suggested that a slow renaturation of calcineurin preceded calmodulin binding. The renaturation process was shown to be dependent on calmodulin and calcium. Following calmodulin-induced renaturation, the binding of radiolabeled calmodulin showed the characteristic specificity and calcium dependence. The dissociation constant determined by kinetic and equilibrium methods was in the nanomolar range similar to that of native calcineurin. Isolated calcineurin A, also bound calmodulin with a similar binding constant and limited proteolysis with trypsin abolished the ability of calcineurin to bind calmodulin in agreement with previous findings.

The availability of this direct binding assay provides us with the means to use the monoclonal antibodies as probes to further define the structure of the interaction sites and the conformational changes accompanying calmodulin binding and enzyme activation (M. Hubbard).

B. cAMP-dependent Protein Kinase in Drosophila Melanogaster

An independent research project has been initiated by John Foster, holder of a Burrough-Wellcome fellowship. Both types I and II cAMPdependent protein kinase are known to be present in Drosophila. The type II cAMP-dependent protein kinase has been purified to homogeniety and is known to be nearly identical to the mammalian enzyme in kinetic and physical properties. This fact was used to clone the Drosophila catalytic subunit gene employing a 60 residue oligonucleotide probe corresponding to a highly conserved 20 amino acid sequence in bovine catalytic subunit (with Drosophila codon preferences). The feasibility of using the probe was demonstrated by hybridiza-

tion to Drosophila genomic DNA that had been digested to completion with either Hind III or Pst I, run on agarose gels and blotted to nitrocellulose. Strongly hybridizing fragments of 3.9 kb for Hind III and 0.5 kb for Pst I were found. The restriction map of the cloned 3.9 kb fragment is consistent with the genomic southern results: The region of the clone that hybridized with the probe was contained in a 0.5 kb Pst I fragment. Sequence analysis confirmed the presence of the catalytic subunit gene in the clone. Sixty percent of the protein coding sequence has been determined revealing an exceptional homology in the C-terminus third of the bovine and fly enzymes (117 identical residues out of 132).

Cloning genes in Drosophila allows one to take genetic approaches for defining function that are more sophisticated than available methods in mammalian systems. In addition, the clone of the Drosophila catalytic subunit gene can be used to study the control of its transcription. In particular, regulatory proteins that bind to the 5' noncoding region can be detected by their ability to block the progression of exonuclease III digestion thereby permitting to develop an assay which can be used to purify these proteins. Thus we hope to purify the Drosophila homolog of the mouse mutant kin^- gene product (John Foster).

Significance to Biomedical Research and the Program of the Institute:

The proteins being studied are important enzymes in the control of cell metabolism and are regulated by protein-protein interactions. cAMP phosphodiesterase and cAMP-dependent protein kinases are involved in the regulation of cellular processes by cAMP. Calcineurin, as a Ca^{2+} regulated protein phosphatase can modulate the function of many enzymes and other proteins whose activity is regulated by phosphorylation. cAMP and Ca^{2+} mediated phosphorylations are critical for the regulation of cell growth and differentiation. Calmodulin, as a common modulator of these, and many other enzymes, provides a link between cyclic nucleotide levels and Ca^{2+} regulation of cellular processes. The ability of calmodulin to regulate a large number of biological processes represents a novel mechanism of great potential physiological importance. The N-terminal myristylation of calcineurin and of the catalytic subunit of cAMP dependent protein kinase, two important enzymes in cellular regulation may prove to be useful in understanding the role of this postranslational modification in cellular transformation. Myristyl residues, identified at the NH_2 terminus of p60SRC, the gag polyproteins and of gag-onc fusion proteins in mammalian transforming retroviruses may direct the membrane association or translocation of these proteins.

Future Course of Research:

We will continue to study the structure of calmodulin in solution in order to characterize the calmodulin conformers responsible for multiple functions, and to identify and characterize the calmodulin interacting site(s) with its target proteins. Our efforts will be concentrated on the study of the structure-function relationships of calcineurin, and on the elucidation of the biological roles of this protein. We will try to clarify the linkage between the two second messengers, Ca^{2+} and cAMP, by studying the effects of calmodulin and Ca^{2+}

on cAMP-dependent protein kinase and protein phosphatase. These studies will be carried out at the molecular level with purified and characterized proteins and at the cellular level with cells in tissue culture to correlate the in vitro observations with physiological events. Among the model systems in which the role of calmodulin and other Ca^{2+}-binding proteins will be investigated are: the differentiation of neuroblastoma glioma hybrid NG-108-15 and in vitro transformation of cells.

Publications:

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Manalan, A.S., Newton, D.L., and Klee, C.B.: Purification and peptide mapping of calmodulin and its chemically modified derivatives by reverse phase HPLC. J. Chromatog. 326: 387-397, 1985.

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Klee, C.B., Newton, D.L., Ni. W-C., and Haiech, J.: Regulation of the calcium signal by calmodulin. Proceedings of CIBA Foundation Symposium on Calcium and Cell Function 122: 162-170, 1986.

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Newton, D.L., Burke, T.J. Jr., Rice, K.C., and Klee, C.B.: Synthesis and properties of $CAPP_1$ calmodulin. Methods in Enzymology (in press).

Putkey, J.A., Draetta, G.F., Slaughter, G.R., Klee, C.B., Cohen, P., Stull, J., and Means, A.R.: TITLE
J. Biol. Chem. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05244-09 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 chererters 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 Investigetor.) (Name, title, laboratory, and institute affilletion)*

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R. Thayer	Chemist	LB	NCI
S. Contente	Staff Fellow	LB	NCI
G. Humphrey	Guest Researcher	LB	NCI
J. Skowronski	Visiting Fellow	LB	NCI
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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 (lymphocytes) B
☐ (a2) Interviews

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

The goal of this work is to understand the structure and possible function of highly repeated DNA sequences and to elucidate the mechanisms by which such sequences are amplified either in tandem or through transposition to new genomic loci. To understand the maintenance and plasticity of satellite DNA sequences we are studying junctions between these long tandem repeats and low copy number genomic sequences. One particular single copy sequence that is conserved in primates and rodents and is joined to species specific satellite DNA in each species analyzed is being characterized. The extensive and rapid changes that occur in satellite DNA sequence on an evolutionary scale are being applied to taxonomic and evolutionary problems: in particular, evolutionary relationships in the carnivores are being investigated. To understand the significance of the LINE-1 family of interspersed repeats, the potential for some family members to be functional genes and encode a protein is being investigated. cDNA clones representing polyadenylated, cytoplasmic LINE-1 RNA from human teratocarcinoma cells have been isolated; the cDNA sequences have defined a subset of LINE-1 sequences that appear to be specifically transcribed (or processed) in these cells. The subset contains at least 19 different members, most of which do not have completely open reading frames and therefore could not encode a full length LINE-1 protein.

GPO 914-918

Project Description

Objectives:

The detailed structure, organization and function of the highly repeated DNA sequences in the primates and other mammals are being studied. These sequences fall into three groups: satellites are long tandemly repeated segments generally concentrated at centromeres and telomeres, SINES are short repeated segments dispersed throughout the genome, and LINES are long repeated segments also dispersed throughout the genome. We are interested in characterizing the structure and function of such sequences. Recently it has become evident that many of these sequences are changeable in position and organization within DNA. They also undergo reactions which bring the sequences of the various family members into conformity within the genome of a particular species (concerted evolution). We are interested in studying the mechanisms as well as the time course and frequency of such changes in order to understand better the fluidity of primate genomes.

Methods Employed:

DNA is isolated from fresh frozen tissue and from cells grown in tissue culture and purified by standard procedures. DNA structural analysis includes the use of a variety of enzymes, including restriction endonucleases, direct DNA sequencing methods (chemical procedure of Maxam and Gilbert and dideoxynucleotide method), radioisotope tracers, gel electrophoresis, and centrifugation. A variety of nucleic acid hybridization techniques, both in solution and on nitrocellulose are used. DNA fragments and RNAs are purified and amplified by molecular cloning in E. coli K12 host-vector systems. Genomic organization is studied by cloning, blotting and hybridization. Functional aspects focus first on transcription both in vivo and in vitro, on translation into protein, and on immunological techniques. For this work RNA is isolated from cells and cloned recombinant cDNA libraries are constructed. Finally, recombinant DNA vectors are constructed and transfected into animal cells to study expression. For chromosomal mapping, somatic cell hybrids constructed by standard techniques are used as is in situ hybridization. Antibodies to synthetic peptides and eukaryotic polypeptides synthesized in E. coli are prepared and characterized by standard techniques. To analyze for sequence specific DNA binding proteins in extracts of monkey and human cells two assays are used. One depends on the protection of the DNA sequence from digestion with exonuclease III (exonuclease footprinting) and the other on protection from digestion by DNase I (DNase footprinting). Extensive use is made of computer analysis of DNA sequences. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research.

Major Findings:

A. Junctions Between Satellite DNA and Low Copy Number Genomic Sequences.

Previously, we demonstrated that an African green monkey (AGM) centromeric satellite DNA called deca-satellite is highly polymorphic in individual

members of the species. This, together with the fact that no function has been assigned to satellite DNA in spite of years of theoretical and experimental analysis, lead us and others to suggest that the generation and maintenance of long tandem repeats of DNA is a by-product of DNA replication and/or recombination. This in turn suggests that these genomic processes may have special features in those genomic regions where satellite DNA recurs regularly, centromeres and telomeres. For this reason we undertook to isolate and study the low copy number genomic sequences that border satellite DNA in primate and other mammalian genomes. In the past year, work from other labs has confirmed the fluidity of satellite structure among individuals of several species, indicating that our studies are likely to have significant generality. Such a conclusion is also supported by the work described below; we have found that a low copy number sequence that borders the AGM deca-satellite is conserved in other mammals both in sequence and in location close to species-specific satellite DNAs.

Last year we reported the isolation of two recombinant phage containing overlapping regions of identity that represent low copy number sequence in the AGM genome. One of these, called λM8, is almost entirely low copy number sequence and the segment in λM8 can be aligned with AGM genomic sequences both as to restriction endonuclease maps and cross-hybridization. A portion of this low copy number sequence (called LCNS-A) also occurs in the second recombinant phage, λJA1, but in this phage the LCNS-A is joined to about 4 kbp of deca-satellite. Present between the satellite and LCNS-A in λJA1 is about 1.5kbp of low copy number sequence that does not appear in λM8 where it is replaced by a non-homologous 2.2kbp region. All of the genomic blots indicate that the monkey genomic sequences closely homologous to LCNS-A are as they are in λM8, including the 2.2kbp region. To summarize, there are two cloned segments that have, in part, identical sequences but only one of them appears to correspond to the genomic organization. What then is the origin of the segment cloned in λJA1? Two explanations have been considered. One is that the segment in λJA1 represents a rearrangement that occurred either in the monkey genome or during cloning in E. coli. The second is that there is more than one copy of the LCNS-A in the AGM genome. Two approaches to these questions have been taken. First, the copy number of two sub-cloned segments from within LCNS-A, p2.2 and p2, were determined by quantitative dot-blot analysis. The p2.2 segment occurs in λM8 but not in λJA1 while the p2 segment occurs in both phage. The results indicate that both fragments occur only once in the AGM genome and therefore are consistent with the existence of only one genomic arrangement of LCNS-A in the monkey genome. The second approach involves additional DNA-blotting experiments with AGM DNA, using additional sub-cloned regions of LCNS-A. All of these results are again consistent with a single genomic arrangement of the sequences. Additional experiments using as a probe the 1.5kbp region unique to λJA1 are in progress. The most likely interpretation of these results seems to be that during cloning, a large deletion involving the 2.2kbp region eliminated this segment in λJA1. Nevertheless, it remains possible that there are two copies of LCNS-A in the AGM genome, copies that are almost identical and perhaps in tandem. The experiments in progress should give a definitive interpretation.

The LCNS-A originally cloned from AGM DNA is conserved intact for at least 7 kbp in human DNA. As reported last year, 14 out of 15 phage selected from a human genomic recombinant library with the monkey LCNS-A anneal to human satellite DNA, indicating that both the sequence and its position joined to satellite is conserved in the two primates. Two of the phage, one with satellite, λHs24, and one without, λHs6, have been characterized in detail. As a pair, they are analogous to the two monkey phage, λJA1 and λM8, respectively. λHs24 is similar to λJA1; it contains human satellite sequences and is missing part of LCNS-A. The missing region includes the 2.2kbp region and also extends approximately 700bp further into the LCNS-A. λHs6 contains the 2.2kbp region and the rest of the LCNS-A intact. We do not yet know if λHs6 or λHs24 contain sequences homologous to the 1.5kbp region in λJA1. As in the case of the monkey clones and genome, the human genome contains the LCNS-A intact, as it occurs in λHs6. This was demonstrated by genomic blotting experiments with a variety of restriction endonucleases having both 4 and 6 bp recognition sites and with about 30 different DNAs isolated from cell lines or lymphocytes. In addition, several subcloned probes from within LCNS-A were used. Notably, in only one of the samples was there a single polymorphism observed with one enzyme. In all cases, a simple hybridization pattern was observed. Thus the data indicate that the LCNS-A sequences are organized in the human genome as they are in λHS6 and in monkey DNA. Moreover, all the data were consistent with a single copy of LCNS-A in the human genome. The copy number was confirmed by quantitative dot blot analysis using human placental and lymphocyte DNA and both the p2.2 and p2 probes. These data are remarkably similar to those obtained for the monkey. It seems likely that the human DNA cloned in λHs24 represents a rearrangement of genomic DNA that involved a deletion of sequences between the satellite and the LCNS-A. In λHs24 this deletion seems to have extended further into the LCNS-A than was the case with λJA1. Altogether the data suggest that the junction between satellite DNA and the LCNS-A is especially susceptible to recombination in E. coli. It will be interesting to learn if this susceptibility also occurs in primate genomes.

In collaboration with O.W. McBride and Mary Harper, the LCNS-A sequence has been mapped to human chromosome 4 both by DNA-blot analysis of human/mouse and human/Chinese hamster somatic cell hybrids and by in situ hybridization to mitotic human chromosomes.

DNA-blotting experiments with genomic DNA indicate that the LCNS-A isolated from AGM is conserved in mice and Chinese hamsters. A total of nine recombinant phage have now been isolated from two mouse genomic libraries using sub-cloned regions of LCNS-A as probes. Plaques representing all nine of the phage anneal with a mouse-specific satellite DNA probe. Thus it appears that the proximity of the LCNS-A sequence to satellite DNA found in primates is conserved in rodents.

B. The LINE-1 Family

The LINE-1 family is a large set of dispersed repeated sequences. Homologous families occur in all mammals. The longest family members are between 6 and

7 kbp (about 6 kbp in primates) and other family members are truncated (at the designated 5'-end) to various extents and/or internally rearranged. The central region of many family members, 4 to 5 kbp, contains lengthy but broken open reading frames and it is this region that is most highly conserved among different species and orders of mammals; in contrast, the 5'- and 3'-ends tend to diverge in different species although the sequences are quite conserved within a species. Thus, concerted evolution appears to homogenize intraspecies family members. The open reading frames, along with the presence of a polyA-rich tail at the 3'-end of LINE-1 units and the target site duplications that flank many LINE-1 units, lead to the hypothesis that LINE-1 is a family containing a few functional genes and a large number of processed pseudogenes. To test this hypothesis, we initiated a search for a polyadenylated cytoplasmic transcript of a full length LINE-1 unit. As reported last year, such an RNA was found uniquely in a human teratocarcinoma cell line, NTera2D1. This RNA represents the strand having the open reading frames and its presence in NTera2D1 cells correlates with the embryonal carcinoma morphology typical of densely packed cells, but not other morphological forms.

Primer extension experiments have now been carried out on the cytoplasmic polyadenylated RNA from the NTera2D1 cells. The bulk of the RNA initiates at the position previously identified as the first common base in full length genomic copies of primate LINE-1 sequences. This finding is of two-fold significance. First, it is consistent with the idea that those genomic LINE-1 sequences that are transcribed and processed into cytoplasmic, polyadenlylated RNA in NTera2D-1 cells share common transcriptional regulatory sequences. Second, it is consistent with the idea that the bulk of genomic LINE-1 sequences represent processed pseudogenes and thus may arise through a process involving reverse transcription.

A cDNA library in the vector λgt10 was prepared from cytoplasmic, polyadenylated NTERA2D1 RNA. Approximately twenty independent clones, each containing between 4.5 and 6 kbp of LINE-1 sequence and the central EcoRI site typical of most family members were selected and are being characterized by primary nucleotide sequence analysis. Thus far, sequence analysis of the 3'-most 2.5kbp of all has been carried out. Several conclusions can be drawn from the sequence data. First, the cDNAs (and the RNAs from which they derive) represent a special subset of genomic LINE-1 sequences because the consensus sequence of their 3'-regions (210bp) is different from the consensus sequence of random genomic clones. Second, because a specific subset of LINE-1s are transcribed, it is likely that they will be associated with some common transcriptional regulatory sequences, as already suggested above. Second, the divergence of the cDNA clones from one another, most notably in the 3'-noncoding region, is much lower than the divergence of randomly selected genomic sequences. Thus, homogenization of this subgroup seems to be specialized. Finally, the majority of the cDNAs do not have completely open reading frames in the 3'-most 2.5kb; the reading frames are broken by single base pair changes and small deletions and insertions. Several of the cDNA's do have completely open reading frames in this region and could encode a polypeptide (795 amino acids). Until the sequence analysis of the 5'-portion of the clones is determined we will not know whether any of the cDNAs

can represent mRNAs that could produce a protein corresponding to a full length LINE-1.

The presence of a LINE-1 family in the species Felis catus has been demonstrated. One, randomly cloned, family member (L1Fc-FaS1) has been sequenced. It contains a large inversion and several insertions, as has been found in other species. Also, it is about 70 percent homologous to the primate LINE-1 consensus sequence in the coding region near the 3'-end and hybridizes to a subset of LINE-1 sequences in both monkey and mouse genomes.

C. Satellite DNA

The rapid evolution of satellite sequences indicate that these DNA segments may be particularly useful for studies in mammalian evolution. For this reason, we are investigating satellite DNA in various species in the order Carnivora. This order was chosen because DNA samples are available for almost all the species within the order and because the evolutionary relationships between the genuses and species are fairly well known. A satellite from the species Felis catus within the family Felidae has been cloned. The sequence was shown by in situ hybridization to be mainly telomeric and to occur on many chromosomes. This satellite, FA-SAT, also occurs in 27 other species of cats although the amount and sequence varies extensively. A total of 21 FA-SAT monomers (483bp) cloned from F. catus have been fully or partially sequenced. There is, on the average, 1.2 percent divergence between monomers suggesting a common sequence about 2 million years ago. Notably, in the consensus 483bp two of the six possible reading frames are open except for a single stop in each. A satellite (CA-SAT) has also been cloned from Canis familiaris. In situ hybridization shows this to be centromeric and the repeat unit is 750bp; a number of monomers have been partially sequenced. Fourteen additional species of canids were examined for the presence of CA-SAT; homologous sequences are present in all 14 and in roughly similar amounts. These data indicate that great variability in satellite sequence and organization can occur over very short evolutionary times (several million years) in some families (cats), but does not occur in all families (dogs). It appears that the sequences can then be used for taxonomic purposes. Among the cats, for example, some animals such as puma or lynx have very litte FA-SAT and the small South American cats have a characteristic dimeric FA-SAT pattern suggesting that they belong to a monophyletic group.

D. SINES

A SINE family (FaS1) unique to carnivores has been isolated and characterized from Felis catus. The sequence is 205bp long, is terminated by a polyA-rich sequence, is bracketed by 16bp direct repeats, has a typical RNA polymerase III A-box and, just preceding the A-rich segment, has a run of 10 CT dinucleotides. This sequence is organized in different ways in different canid species and can be used to distinguish between them.

To assess the possible physiological role(s) of the interspersed, repeated primate Alu sequence family we have examined cloned Alu family members for

the presence of binding sites for sequence-specific DNA-binding proteins. For this study, a DNA restriction fragment containing a representative Alu sequence derived from the African Green monkey genome (designated p7.01) was end-labeled with T4 polynucleotide kinase at either end, then incubated with an extract of BSC-1 (monkey) cell nuclei and digested with Exonuclease III. We observed exonuclease stops bracketing a 25 base pair region of the Alu sequence indicating the presence of protein bound at a specific site. This site was located between the regions of A-box (pol III promoter) and SV40 ori homology in the second (3') Alu monomer. No stops were observed in the homologous, but not identical, region of the first (5') monomer. We are currently examining other Alu sequences from African Green monkey and humans to see if the factor in BSC-1 nuclei binds to the homologous site. We also detected an Alu-sequence binding material in extracts from the CV-1 monkey cell line and the human HeLa S3 and NteraD2D1 cell lines. Binding activity was not detected in human adult liver tissue. We have employed HeLa S3 cells as a source of Alu-sequence binding protein for purification. The protein was extracted from isolated nuclei at 0.5 M NaCl and chromatographed on heparin-Sepharose (eluted at 0.25-0.4 M NaCl) followed by DEAE-cellulose (eluted at 0.13-0.15 M NaCl). Using this partially purified protein, we obtained a DNAse I footprint at the binding site.

Future Course of the Work:

A. Junctions Between Satellite DNA and Low Copy Number Sequences.

Because of the complexities encountered in establishing the genomic organization of the LCNS-A in primate genomes, the functional studies planned for this year were postponed. Some structural data remain to be obtained, including additional genomic mapping with additional probes, determination of the locus of the LCNS-A on human chromosome 4, and primary nucleotide sequence data that can further elucidate the nature of the rearrangements common to cloned segments containing both satellite and LCNS-A. After this is completed we will proceed with the functional studies proposed in last year's report. Also, we plan on resuming studies on two additional satellite-low copy number junctions that have been cloned but not extensively characterized.

B. The LINE-1 Family.

Our central goal is to determine whether one or more of the members of the LINE-1 family represents a functional gene. Characterization of the cDNA clones from the NTera2D1 RNA will be completed. Primarily, this involves sequence analysis of the 5'-portions. Segments from the cDNAs that have open reading frames will be inserted into expression vectors for synthesis of the corresponding polypeptides in E. coli and these polypeptides will be used to prepare antibodies. Previously prepared antibodies against short synthetic peptides representing LINE-1 coding sequences have given uninterpretable results. The antibodies will then be used to try and identify and localize proteins encoded by LINE-1 in NTera2D1 cells. If the antibodies prove effective, we will try to detect cross-reacting material in early mouse embryos. Because many of the cDNAs appear to represent RNAs that can not be

translated into long polypeptides, it is important to determine whether any of the RNAs are capped and associated with polysomes in the cells.

Analysis of the cDNAs has indicated that a subset of LINE-1 elements are transcribed in NTera2D1 cells. A functional gene, if it exists, should be within this subset. There are sufficient sequence differences between the subset and random genomic LINE-1 elements to allow specific selection of the transcribed subset from a genomic library. Isolation of such clones should lead to the characterization of transcriptional regulatory elements.

Recently it has become evident that the protein predicted from the open reading frame in LINE-1 has homology to retrovirus and retrotransposon reverse transcriptases. Using the techniques successfully applied by Steven Goff for the production of active retrovrus reverse transcriptase in E. coli, we will test the hypothesis that LINE-1 encodes a reverse transcriptase. The feline LINE-1 family will be further characterized.

C. Satellite DNA

We will continue to investigate the feasibility of using satellite DNA sequences to resolve taxonomic questions and to illuminate evolutionary processes.

D. SINES

The purification of the putative Alu-specific binding protein will be pursued. In particular, we will attempt further purification using a synthetic oligonucleotide representing the binding site attached to a cellulose matrix. The specificity of the binding reaction will be studied.

Significance To Cancer Research:

The critical role of genome organization and reorganization in tumorigenesis has become more and more apparent in recent years. For this reason, fundamental understanding of the organization of normal genomes is essential to understanding oncogenesis. Of particular interest is the fact that highly repeated DNA sequences, whose genomic function is unknown, are major sources of genomic reorganization. Through an understanding of their source and contribution to recombination, transposition and amplification, processes important to tumorigenesis can be elucidated. A particularly interesting recent example is the fact that a LINE-1 element has been found to interrupt the myc locus in a canine tumor. Thus, transposition of LINE-1 may be the cause of changes in gene expression that contribute to the tumor phenotype. Similar events may occur with SINES, which are also moveable genomic elements. In addition, the growing appreciation of the enormous fluidity of satellite DNA sequences even at the level of individual organisms, a phenomenon described several years ago by this laboratory, emphasizes the role that these sequences might play in chromosomal events and rearrangements.

Publications:

Singer, M.F.: Genetics and the law: A scientist's view. Yale Law and Policy Review, Vol III, No. 2: 315-335, 1985.

Dynan, W.S., Saffer, J.D., Lee, W.S., and Tjian, R.: Transcription factor Sp1 recognizes promoter sequences from the monkey genome that are similar to the simian virus 40 promoter. Proc. Natl. Acad. Sci. USA 82: 4915-4919, 1985.

Skowronski, J., and Singer, M.F.: Expression of a cytoplasmic LINE-1 transcript is regulated in a human teratocarcinoma cell line. Proc. Natl. Acad. Sci. USA, 82: 6050-6054, 1985.

SenGupta, D.N., Zmudzka, B.Z., Kumar, P., Cobianchi, F., Skowronski, J., and Wilson, S.H.: Sequence of human DNA polymerase β mRNA obtained through cDNA cloning. Biochem. Biophys. Res. Commun. 136: 341-347, 1986.

Lee, T.N.H., and Singer, M.F.: Analysis of LINE-1 family sequences on a single monkey chromosome. Nucleic Acids Res. 14: 3859-3870, 1986.

Skowronski, J., and Singer, M.F.: The abundant LINE-1 family of repeated DNA sequences in mammals: Genes and pseudogenes? In 51st Symposium on Quantitative Biology, New York, Cold Spring Harbor Laboratories (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05258-07 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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	Z. Lin	Visiting Fellow	LB	NCI
J. Eldridge	Biochemist	LB	NCI	J. Hammer	Guest Researcher	LB	NCI
A. Levi	Visiting Associate	LB	NCI	B. Winter	Visiting Fellow	LB	NCI
J. Rodriguez	Guest Researcher	LB	NCI				
W. Quitschke	Visiting Fellow	LB	NCI				
R. Di Lauro	Visiting Scientist	LB	NCI				
L. DePonti-Zilli	Visiting Fellow	LB	NCI				

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
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SECTION
Developmental Biochemistry and Genetics Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	5	0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The level of expression and the appropriate regulation of the various chicken actin genes, when stably introduced into mouse myogenic cell lines, is dependent upon the developmental history of the particular line. The skeletal actin genes are not appropriately regulated in adult myogenic satellite cells, whereas correct regulation is observed in embryonically derived lines. The cytoplasmic beta actin down regulates during myogenesis in all muscle lines tested so far. These various lines were used to demonstrate that all the sequence information necessary for the regulated expression of the actin genes was present in our genomic isolates. Promotor constructs with the bacterial CAT gene have shown the transcriptional control and regulatory regions for cardiac actin are contained within the 300 bp 5' to the start of transcription. Transcription of the beta cytoplasmic actin is not directly regulated through the promotor region, as determined by promotor exchanges and CAT constructs. Deletion analysis has defined a 320 bp region spaning the polyadenylation signal that is responsible for the transcriptional regulation of beta actin. The promotor regions for the myosin LC1/LC3 gene have been defined with CAT constructs. Sequences involved in the regulated, tissue specific expression of these genes are also contained within these promotor regions. Two lower eukaryotic systems are under investigation: the cytoplasmic myosin gene of Acanthamoeba and the myosin gene of yeast. We have isolated and determined the nucleotide sequence of the Acanthamoeba gene and compared its structure and organization to other myosin genes. Using a probe from the myosin gene of C. elegans we have identified homologous sequences in the yeast genome. PC12 cells undergo neuronal differentiation in response to NGF. We have isolated a cDNA sequence and the corresponding gene that is induced 50-80 fold in response to NGF. The cDNA encodes a polypeptide of 72,000 daltons. We have made antibodies to different portions of the cDNA using Lac Z fusions to study the induction and localization of the protein. The promotor of the NGF induced gene responds to NGF when fused to CAT and transfected into PC12 cells.

Project Description

Objectives:

A. To prepare ds cDNA probes for the genes of interest, and to utilize these probes to isolate the genomic sequences for structural and regulatory studies. These studies initially involve sequence analysis of all or part of the transcriptional unit for a given gene.

B. To define the transcriptional start sites, splice junctions and termination signals for the genes of interest. This information will be used in a comparative study of differentiation specific and "house keeping" sequences.

C. Some of the ds cDNA probes are to be used in chromosomal localization studies on mouse chromosomes. Several of the structural genes under investigation are highly conserved across species. It is of interest to know if isoforms are proximal or distal on the same chromosome, or on different chromosomes.

D. When the promotor regions for various genes have been identified, it is intended to analyze promotor function in one of the eukaryotic vector systems now available. We intend to: 1) see if a promotor for a differentiation specific gene, such as α actin, can function when placed in an undifferentiated cell or if differentiation is required for function; 2) define the essential sequence elements for promotor function and regulation; 3) determine the role of the intron arrangement in the regulation of gene expression; 4) examine the role of polyadenylation in the regulation of gene expression; 5) analyze the proteins associated with regulatory regions responsible for modulation of gene expression.

Methods Employed:

Specific ds cDNA probes are prepared either from A^+ mRNA or mRNA fractionated on preparative methylmercury gels. Fractions enriched for a particular mRNA, as judged by analysis in a cell-free protein synthesizing system, are cloned using standard methods. Clones are identified by positive selection and sequence analysis.

The probes are used to screen various genomic libraries with standard procedures. The distribution of the coding information and its polarity within a given isolate is determined by hybridization with kinased RNA and short cDNA. The 5' proximal and 3' proximal fragments in the appropriate restriction digest are sequenced and mapped by primer extension and S1 analysis to clearly determine the end points of the transcriptional unit for each gene. Restriction fragments containing regions of interest are subcloned into one of the vector systems for further analysis.

Major Findings:

Regulation of actin gene expression

Cardiac actin is the major muscle specific isoform expressed in embryonic muscle

and in cultured muscle cells, whereas beta cytoplasmic actin is expressed in all dividing cells but not in muscle tissue. We have introduced the chicken genes for beta and cardiac actin into three different murine myogenic cell lines, each with different developmental histories. The C2 line is derived from adult satellite cells and the F3 and B1 lines are of embryonic origin. In C2 cells stably transformed with the chicken cardiac actin gene, expression is constituative at high levels throughout all phases of myogenesis eventhough the endogenous mouse cardiac actin gene is expressed only in the differentiated muscle cells. In contrast, when the same gene is introduced into either the F3 or B1 cells it regulates in parallel with the endogenous gene. Thus, all the sequence information necessary for the appropriate tissue specific regulation of the cardiac gene is contained in this genomic isolate. Promotor deletions fused to the bacterial CAT gene and transfected into primary cultures of chick embryonic breast muscle have defined a 300 base pair region that regulates transcription and tissue specific expression. This region is being used in protein binding studies to assay factors involved in tissue specific expression using nuclear extracts from the various cell lines and from embryonic chick muscle. The beta actin gene is appropriately regulated in all the muscle cell lines examined: expression of the transfected chicken gene decreases in parallel with the endogenous gene during myogenesis. Nuclear runon studies have shown this regulation is transcriptionally controlled, however, promotor exchange experiments and deletion studies have localized the regulatory region to the 3' end of the beta actin gene in a 300 base pair region spanning the poly A site. Protien binding studies and a detailed deletion analysis are under way.

The myosin light chain gene

The myosin light chains 1 and 3 are encoded by a single gene. The organization of the gene has been determined by detailed restriction analysis, partial sequence, primer extension and S1 mapping. The gene contains 9 exons spanning 18Kb. The transcriptional starts for LC1 and LC3 are roughly 9Kb apart. The transcriptional pattern is developmentally regulated and processing of transcripts involves differential splicing to a common set of 3' exons. The gene contains two adenylation sites which are used randomly in all muscle tissues examined. Initial attempts to study expression with LC promotors fused to the bacterial CAT and introduced into the various mouse muscle lines gave no result suggesting the individual promotors would not function out of the context of the entire gene i.e. a specific enhancer might be involved in light chain expression. Although the LC promotor CAT fusions did not function in mouse cultures, both constructs were very active and developmentally regulated in primary cultures of embryonic chick muscle. S1 analysis has shown the correct promotor starts are being used to produce CAT activity. This differential expression in mouse and chicken cells, and the factors involved in expression in chick cells are under study.

Gene induction by Nerve Growth Factor (NGF)

PC12 cells are derived from the adrenal gland of the rat and differentiate into neuronal cells in culture in response to NGF. We have isolated a cDNA clone and the corresponding genomic sequence that is induced 50 to 80 fold within 24 hours after exposure to NGF. We have determined the entire amino acid sequence of the

polypeptide from the largest open reading frame in the cDNA and have generated polyclonal antibodies to the protein using cDNA-LacZ fusions. Western blots and cell-free translations now indicated two polypeptides are induced with NGF and react with the polyclonal antibodies. The basis for these two polypeptides is under investigation. The antibodies are being used to localize the protein within the cell and to measure protein induction by western blot. Preliminary immunofluorescence studies indicate the protein is diffuse in the cytoplasm of the cell and is specific for the PC12 cell. No other cells of neuronal origin or different cell type cross react with the antibody so it appears to be a marker for a specific neuronal cell type. Although low levels of the mRNA for the NGF induced protein can be detected in the brain, we have not been able to see any immunofluorescence in brain sclices. The promotor for the NGF induced mRNA has been fused to the bacterial CAT gene and does appear to respond to NGF induction when transfected into PC12 cells. It is not induced in other cell backgrounds. This regulation is under investigation.

Characterization of the Acanthamoeba myosin II gene

We have used the 2.7 Kb BamH1 fragment of the C. elegans body wall myosin heavy chain gene to isolate the three Acanthamoeba myosin proteins, myosin IA, myosin IB, and myosin II. The BamH1 fragment contains the coding information for the ATP and actin binding sites. This fragment lead to the isolation of 40 plaque purified phage from an Mbo1 partial phage library. 17 of these phage were characterized by detailed restriction enzyme analysis and were resolved into four non-overlapping groups. Hybrid selection defined the phages encoding the myosin II gene. One of these was sequenced completely and compared to the rabbit and C. elegans proteins. The gene has three exons and considerable amino acid homology with other myosins. Structural features are under analysis.

Studies on yeast structural proteins

The same BamH1 fragment from the C. elegans myosin gene was used to screen various restriction enzyme digests of yeast genomic DNA, with positive results. Preliminary analysis of a yeast genomic library has produced positive plaques and these are under investigation. Yeast has a single actin gene that is essential for normal viability. Gene replacement studies are under way to determine if several of the vertebrate actins can functionally replace the yeast gene since there are specific amino acid differences in the vertebrate actins characteristic for each isoform. The complete cDNA sequences for human alpha, beta, and gamma actins are being used in this study for each has a restricted pattern of expression in the vertebrates that reflects a particular function.

Structure of the pyruvate kinase gene

The structural portion of the chicken pyruvate gene has been defined. 2300 base pairs of coding sequence contained in 10 exons are distributed over 17 to 18 Kb of DNA. Attempts to isolate the 5' noncoding exon of 55 base pairs were not successful. A 42 base pair oligonucleotide representing the 5' noncoding portion of the PK cDNA was used to screen a size fractionated genomic library constructed in lambda GT10. Eventhough the oligo could be used to detect homologous sequence in total RNA and in genomic DNA digests, the sequence was never found in any library tested. Other methods of cloning are under study.

The rat thyroglobulin promoter

A 900 base pair long DNA segment containing the transcriptional start site of the rat thyroglobulin (Tg) gene has been fused to the bacterial gene for chloramphenicol acetyltransferase (Cat). The fusion gene has been introduced into three different cell lines derived from the rat thyroid gland and into a rat liver cell line. Expression of the fusion gene has been detected only in the one thyroid cell line that is able to express the endogenous Tg gene. The minimum DNA sequence required for the cell type specific expression was determined by deletion analysis; it extends 170 nucleotides upstream of the transcription initiation site. The Tg promoter contains three readily detectable binding sites for factor(s) present in salt extracts of thyroid cell nuclei. One of these binding sites is not recognized by the nuclear extracts of any other cell type that has been tested, suggesting that it may help mediate the cell type secific expression of the Tg gene.

Projected Course of Research:

We intend to analyze the organization, structure, and regulation of constituative and differentiation specific genes.

Publications:

Zehner, Z.E., and Paterson, B.M.: The chicken vimentin gene: Aspects of organization and transcription during myogenesis. Annals of the New York Academy of Science, Vol. 455, pp. 79-94, 1985.

Eldridge, J., Zehner, Z., and Paterson, B.M.: The complete nucleotide sequence of the chicken cardiac alpha actin gene: No strong sequence homologies in the promoter and 3'-translated regions with the chicken skeletal alpha actin gene. Gene 36: 55-63, 1985.

Levi, A., Eldridge, J., and Paterson, B.M.: Molecular cloning of a gene sequence regulated by nerve growth factor. Science 229: 393-395, 1985.

Hammer, J.A., Korn, E.D., and Paterson, B.M.: Isolation of a non-muscle myosin heavy chain gene from Acanthamoeba. J. Biol. Chem. 261: 1949-1956, 1986.

Paterson, B.M., Eldridge, J., and Seiler-Tuyns, A.: Regulation of actin gene expression: Analysis of potential regulatory regions. In Emerson, C., Fischman, D.A., Nadal-Ginard, B., and Siddiqui, M.A.R. (Eds.): Molecular Biology of Muscle Development. New York, Alan R. Liss, Inc., 1986, pp. 550-563.

Hatamochi, A., Paterson, B.M., and deCrombrugghe, B.: Differential binding of a CCAAT DNA binding factor to the promoters of the mouse α_2(I) and α_2(III) collagen genes. J. Biol. Chem. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05262-06 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Eukaryotic Gene Regulation and Function: The Metallothionein System

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

Dean H. Hamer	Research Chemist	LB	NCI				
C. Schmidt	Chemist	LB	NCI	C. Seguin	Guest Researcher	LB	NCI
M. Wong	Visiting Fellow	LB	NCI	D. Thiele	Guest Researcher	LB	NCI
A. Leone	Visiting Associate	LB	NCI	C. Wright	Staff Fellow	LB	NCI
M.J. Walling	Microbiologist	LB	NCI				
J. Trnovsky	Visiting Fellow	LB	NCI				

COOPERATING UNITS *(if any)*
None

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Cellular Regulation Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	7.0	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 regulation and function of metallothioneins are being analyzed by molecular genetic and biochemical approaches in both mammalian cells and yeast. A mouse nuclear protein that interacts with the heavy metal control sequences of the mouse metallothionein-I gene has been detected and is regulated by cadmium in vitro. The structural basis for differential metal binding of human metallothionein-I isoform genes has been investigated by gene shuffling and transfer experiments. Regulation of these genes during development and by oncogenes is also being studied. In yeast, trans-acting mutants that alter metallothionein gene regulation have been isolated. A substantial number of mutants have been engineered into the yeast metallothionein coding sequence and are proving useful in determining the role of individual amino acids in its structure and function.

Project Description

Objectives:

We wish to understand how eukaryotic genes are activated or repressed in response to changes in the cellular environment and how the resulting gene products allow the cell to adapt to its altered conditions.

Methods Employed:

Recombinant DNA, biochemical and genetic techniques are used to identify the regulatory DNA sequences and the cellular factors with which they interact and to characterize the physiological roles of the gene products.

Major Findings:

A. Regulation and Function of Mammalian Metallothionein

Metallothionein (MT) synthesis is an especially useful system for studying eukaryotic gene regulation and function. These small, cysteine-rich proteins have been found in all eukaryotes examined, ranging from yeast to man, and are expressed in many different organs and cell types. MT gene expression is inducible by the same heavy metals to which the MT proteins bind. This homeostatic regulatory mechanism plays a critical role in detoxifying toxic ions, such as cadmium and mercury, and may also play a role in metal homeostasis. The metallothionein genes are also regulated during development in higher organisms, and in man there are multiple MT isoforms that are expressed in a cell-specific manner.

1. Nuclear factors that bind to metal control sequences

We previously demonstrated, by mutagenesis and gene transfer experiments, that a short DNA sequence present in multiple copies 5' to the mouse MT-I gene is responsible for its inducibility by heavy metals. In order to identify cellular factors that might bind to this sequence, nuclear extracts were prepared from uninduced or cadmium-induced mouse cells and analyzed by the exonuclease-III footprint technique. The induced cell extract, but not the control extract, specifically protected one of the metal responsive DNA sequences. To test whether this was due to increased synthesis or increased DNA binding of the factor, extracts from noninduced cells were treated with cadmium and tested by the footprint assay. The *in vitro* treatment was as effective as the *in vivo* treatment in inducing binding to the regulatory sequence. These results suggest that cadmium induces MT gene transcription by increasing, either directly or indirectly, the affinity of a nuclear factor for the metal regulatory sequence. Oligonucleotides corresponding to the regulatory sequence have been synthesized and are being used to affinity purify the factor.

2. In vitro transcription

Elucidation of the mechanism of MT gene regulation will ultimately

require purified *in vitro* transcription systems. As a first step, we have developed a mouse cell nuclear extract capable of faithful transcription of the mouse MT-I gene. This system is being used to compare the DNA sequences required for efficient transcription of the gene *in vitro* as compared to *in vivo* and to test the effect of nuclear factors as described in part 1.

3. Structural basis of differential metal binding

The human MT-I_E and MT-I_F isoform genes are expressed in a tissue-specific, generally reciprocal fashion. To test the idea that these proteins might play differential roles in metal metabolism, we engineered mouse cell lines that overproduce one or the other of these isoproteins. These isogenic cell lines were then tested for their ability to accumulate Cd, Zn and Cu when grown in various concentrations of these metals. We found that cells expressing the MT-I_F protein sequester a 6-fold larger ratio of Cu to Zn than do cells expressing MT-I_E. Exon shuffling experiments how that this effect is dominated by the carboxy domain of the proteins and efforts are underway to identify the precise amino acid change involved. These results suggest that different tissues express different MT genes to serve specific individual roles in metal metabolism.

4. Mechanisms of regulation during development and by oncogenes

The endogenous hMT-I_E and hMT-I_F genes are regulated in a cell-specific manner that can be perturbed by the action of activated Ha-*ras* oncogenes. In an attempt to determine whether these forms of regulation operate in *cis* or in *trans*, cloned hMT-I_E and hMT-I_F fusion genes have been introduced into various transformed and normal human cell lines. Our preliminary results suggest that the hMT-I_E and hMT-I_F genes are repressed during development by fundamentally different mechanisms.

B. Yeast Copperthionein Model System

The yeast *Saccharomyces cerevisiae* synthesizes a small, cysteine-rich protein that binds copper ions. The synthesis of this protein, which is encoded by the *Cup1* locus, is dramatically increased when copper is added to the culture medium. The tremendous genetic and technical advantages of yeast as an experimental organism make this an attractive model system to study the function and regulation of a metallothionein-like protein.

1. Transcriptional regulatory genes

The copper induction of *Cup1* gene transcription is controlled by a short, duplicated upstream DNA sequence. We are using a genetic approach to try and isolate proteins that interact with this positive control element. A large collection of copper-sensitive mutants has been generated from which two strains that fail to properly regulate their endogeneous *Cup1* gene were detected. These strains also fail to induce an episomal *Cup1-galK* fusion gene demonstrating that the mutation

effects Cup1 transcription in trans. We are now attempting to map these mutations and to isolate the corresponding genes by complementation.

2. Structure-function studies

Purified yeast metallothionein is a 54 amino acid peptide that contains 8 atoms of coppper and that lacks the first 8 amino acids predicted by the DNA sequences. A series of mutations have been introduced into the Cup1 structural gene to pinpoint amino acid residues that contribute to its structure and function. The structural studies are being performed in close collaboration with Dennis Winge at the University of Utah.

We initially concentrated on the unusual NH_2 terminus of the molecule. Mutants were constructed that (1) change residues 2 and 3 just downstream of the initiator met; (2) change residues 8 and 9 at the cleavage site; or (3) delete amino acids 2 to 8. Class (1) mutants have no effect on cleavage. Class (2) mutants completely block cleavage at the normal site and lead to the synthesis of both full length molecules (80%) and a 58 amino acid species (20%). Class (3) mutants synthesize the expected 55 amino acid species with no cleavage step. None of these mutants has any discernible effect on the ability of metallothionein to chelate copper either in vivo or in vitro. An antibody was raised to yeast metallothionein and used for immunofluorescence studies. Both wild-type and mutant proteins appeared exclusively cytoplasmic. The full-length protein produced by class (2) mutants is being used for structural and proteolytic analysis of the NH_2-terminal region.

More recently, we have begun to test the function of the copper-binding cysteine residues of the protein. Four different truncations of the molecule have been engineered, and for each a corresponding double cysteine change has been introduced. Preliminary studies indicate that these alterations decrease the copper-binding properties of the protein both in vivo and in vitro. Mutations at different sites have different quantitative effect, possibly corresponding to he coordination of the sulfurs to common versus distinct copper ions. Results from these initial double cysteine changes should serve to direct the design of further single site mutants.

Publications:

Hamer, D.H., Thiele, D.J., and Lemontt, J.F.: Function and autoregulation of yeast copperthionein. Science 231: 685-690, 1985.

Winge, D.R., Nielson, K.B., Gray, W.R., and Hamer, D.H.: Yeast metallothionein. J. Biol. Chem. 260: 14464-14470, 1985.

Thiele, D.J., Walling, M.J., and Hamer, D.H.: Mammalian metallothioneins are functional in yeast. Science 231: 854-856, 1986.

Thiele, D.J., and Hamer, D.H.: Tandemly duplicated upstream control sequences mediate copper-induced transcription of the yeast copper-metallothionein gene. Mol. Cell. Biol. 6: 1158-1113, 1986.

Schmidt, C.J., and Hamer, D.H: Cell specificity and an effect of ras on human metallothionein gene expression. Proc. Natl. Acad. Sci. USA 83: 3346-3350, 1986.

Hamer, D.H.: Metallothionein. Ann. Rev. Biochem. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05263-05 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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
Barbara Walker	Staff Fellow	LB	NCI
Susan Wilson	Research Assistant	LB	NCI
Vincenzo Zimarino	Visiting Fellow	LB	NCI
Hitoshi Ueda	Visiting Fellow	LB	NCI
Barbara Davis	Laboratory Worker	LB	NCI

COOPERATING UNITS *(if any)*
None

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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:
5.5	5.0	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.)*

Using a new exoIII footprinting assay, we have purified Drosophila heat shock gene activator protein to homogeneity. The protein migrates with a mass of 110 kilo daltons on denaturing gels; its binding activity is detectable within minutes of heat shock. The binding occurs with high affinity ($K_D = 4 \times 10^{-12}M$) to the heat shock control sequence. The exoIII footprinting technique has been applied successfully to the Drosophila heat shock gene TATA box sequence, and to the rat insulin II gene and the Drosophila segmentation gene fushi tarazu.

GPO 914-918

Project Description

Objectives:

A knowledge of gene regulation is fundamental to an understanding of eukaryotic development and differentiation. We wish to understand general principles of gene regulation through an analysis of specific protein-DNA interactions in vivo and in vitro. We have developed nuclease digestion techniques using Drosophila heat shock genes as a model system, and have found two coincident structural indicators of specific protein-DNA interactions in chromatin: hypersensitivity to DNase I cleavage, and resistance to exonuclease digestion. We aim to analyze further the nature and interactions of these specific proteins and DNA sequences.

Major Techniques Employed and Major Findings:

We have developed an exonuclease protection technique for mapping sequences in free DNA on which regulatory proteins are bound. The technique involves the incubation of a 5' end-labeled DNA fragment with a crude 0.35M NaCl nuclear extract followed by exonucleaseIII digestion. After electrophoresis on a sequencing gel, the size of the resistant fragment(s) indicates a border of specific protein-binding.

Using this exoIII "footprinting" assay we have found that the heat shock (hs) activator protein protects sequences from -52 to -91 upstream of the Drosophila hsp82 gene, in good agreement with previous results which showed binding to chromatin from -50 to -86. We have employed this assay to fractionate hs activator from 0.35M NaCl nuclear extracts of heat shocked Drosophila tissue culture cells. From 30 liters of cells (1.5×10^{11} cells), we have been able to purify hs activator to greater than 95% homogeneity (7% yield) after 3 chromatographic steps: heparin-Sepharose, synthetic heat shock element coupled to Sepharose (affinity chromatography), and FPLC Mono S. Purified hs activator migrates with a mass of 110 kilodaltons on a SDS polyacrylamide gel. Susan Wilson now routinely isolates about 1 microgram of hs activator per month.

The exoIII footprinting technique permits the possible identification of DNA-binding proteins specific for virtually any gene which has been cloned. We have applied the technique to the analysis of the Drosophila heat shock gene TATA box and have successfully identified an activity in (unshocked) Drosophila extracts which binds specifically to the hsp70 TATA box. Barbara Walker has confirmed and extended these results. She has subjected nuclear extracts to chromatographic fractionation using phosphocellulose, hydroxylapatite, heparin-Sepharose and Sephacryl S200 and S300 gel filtration columns. She has found that the TATA binding activity fractionates very poorly on all columns tested with significant decrease in recoverable activity. She has also found that the TATA box binding activity may be associated with hs activator due to co-elution of both activities on some columns. Finally she has provided a possible explanation based on single-stranded DNA Fold-back that may account for the specific exoIII stops seen on naked DNA that are smaller than expected half-molecule size.

Vincenzo Zimarino has used the footprinting technique to show that hs activator may be converted from a non-binding to a specific DNA-binding form within minutes of hs stimulation, and that other inducers of heat shock genes such as dinitrophenol also stimulate this conversion. Thus our studies of the mechanism of heat shock gene activation have been pushed one level upwards to a presumed critical modification which switches on the heat shock activator in response to stress. In separate footprinting experiments with mammalian extracts, he has also shown that the rat insulin II gene promoter region contains three binding sites for protein; two of these sites are protected by extracts of non-insulinoma cell extracts, implying that both general and specific factors mediate the expression of the insulin gene.

Hitoshi Ueda has found at least four binding sites within the first 900 bp of the Drosophila segmentation gene fushi tarazu (ftz). Three of these activities are seen in extracts of 5-9h embryos and one is found predominantly in 12-24h embryos, when the ftz gene is turned off. He is starting to fractionate the last activity from kilogram amounts of Drosophila embryos which are collected from our Drosophila mass population.

Barbara Davis is continuing her superb maintenance and management of the Drosophila mass population which should see increasing use by us and others at the NIH.

Significance to Cancer Research:

Our work contributes to a broad effort in the study of gene regulation during normal development and differentiation, and will serve as a basis for the study of aberrant cellular functions which result in neoplasia.

Proposed Course of Research:

We plan to:
(1) Generate mouse antibodies to hs activator, and
(2) obtain a partial sequence from purified hs activator, as a prelude to cloning the gene.
(3) Study the conversion of hs activator from the non-binding to specific DNA-binding form.
(4) Demonstrate that purified hs activator can stimulate transcription in vitro.
(5) Continue studies on the TATA-box binding protein.
(6) Wrap up the footprinting experiments with the rat insulin II gene.
(7) Continue and extend the footprinting experiments with the Drosophila ftz gene.

Publications:

Wu, C.: An exonuclease protection assay reveals heat shock element and TATA box DNA-binding proteins in crude nuclear extracts. Nature 317: 84-87, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05264-05 LB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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 personnal 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	1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Studies dealing with the structure and function of the type II IAP elements were continued. These elements can be distinguished from the more abundant type I IAP elements by a specific 270 bp insertion designated AIIins. Sequencing of several type II IAP elements revealed that part of the region coding for retroviral endonuclease is duplicated and has made it possible to isolate and clone sequences containing only AIIins for use as a specific probe for these elements. Using this probe to analyze Southern blots of mouse genomic DNA, we have shown that: (a) association with other repetitive sequences appears to be a general property of many typeII IAP elements; (b) amplification of type IIB elements in MOPC-315 myeloma is 6-fold and in MOPC-104E myeloma 2-fold relative to embryo DNA; (c) partial demethylation of type II elements in myeloma cells correlates with the known transcriptional activity of the element subclasses. We propose that association with L1 repeats is a feature of transcriptionally silent type II IAP elements, and may reflect their location in a particular chromosomal environment. In situ hybidization of this probe to mouse metaphase chromosomes revealed that the type II elements are clustered on a limited number of chromosomes.

Project Description

Objective:

To study the consequences of the presence of repetitive sequences in the genome, particularly the relationship between the reiterated endogenous retrovirus-like elements of the IAP gene family and normal development and/or neoplastic transformation. Specifically, to determine whether type II IAP genes have a functional role in mouse cells and are expressed as protein products.

Methods Employed:

Culture of animal and bacterial cells; recombination and cloning of specific eukaryotic and viral sequences in plasmids, lambda phage; analysis of DNA components by restriction endonuclease cleavage, electrophoresis, and blot hybridization; isolation of DNA by CsCl banding; electron microscopy of DNA heteroduplexes; nucleotide sequencing by the Sanger dideoxy method; transfection of animal cells with cloned DNA using the CaP precipitation procedure; S1 mapping of transcripts; in vitro RNA transcription; translation in rabbit retriculocyte lysate system; immunoprecipitation; analysis of proteins on SDS-acrylamide gels.

Major Findings:

A. Isolation of a Type II IAP Element Specific Probe.

Sequencing of a number of type II IAP elements isolated from a mouse genomic library defined a type II specific insertion of 270 bp which we designated AIIins. This sequence was considerably shorter than the 500 bp fragment previously believed to be a specific probe for this insertion; the latter was composed of nearly 50% type I IAP sequences.

B. Determination of the Number of Type II IAP Elements in the Mouse Genome.

Hybridization of the AIIins specific probe to Southern blots of mouse DNA was used to determine the numbers of type II IAP element copies of each class (A,B,C defined on the basis of the sizes of internal sequence deletions). DNAs from several myelomas known to contain amplified type IIB elements were compared with DNA from sperm after cutting the DNAs with two restriction enzymes which have conserved sites at the ends of the elements. The three subclasses are divided into EcoRI/HindIII fragments of 3.7, 2.8 and 2.1 Kb by this digestion. After hybridization to the AIIins probe, the blots were exposed to film which was scanned to quantify the amount of material in each component. We estimate that there are 400 copies of type II IAP elements in mouse embryo DNA, with the subclass copy number distribution as follows: 180 IIA, 110 IIB, and 90 IIC. Amplification of IIB elements was 2-fold in MOPC-104E myeloma DNA and 6-fold in MOPC-315 myeloma DNA. These numbers are higher than previously estimated by others using the non-specific probe.

C. Methylation of Type II IAP Elements Correlates with Transcriptional Activity.

It is known that type I IAP transcripts are generally abundant in myeloma cells and that transcription of these elements correlates with demethylation of the sequences in genomic DNA. Several myelomas express primarily type II IAP elements of the IIB subclass. The methylation state of the type II IAP sequences was therefore examined in DNAs from two myeloma cell lines and from embryo. The DNAs were cut with HpaII or MspI and analyzed with the AIIins probe after Southern blotting.Type II IAP sequences in DNA digested with MspI (unaffected by methylation) were resolved into fragments of 2.1 (A), 1.5 (B) and 1.4 (C) Kb. The 5' cut generating these fragments is in the IAP 5' LTR and demethylation of this LTR site is important for its promoter activity. None of these fragments was seen in the HpaII (sensitive to methylation) digest of embryo DNA. In myeloma DNAs the 2.1 and 1.5 Kb fragments were evident, and the 1.5 Kb HpaII fragment from the IIB elements was especially prominent in MOPC-315 DNA, consistent with transcription of these elements into abundant RNA and with amplification of the DNA sequences in these cells. No demethylation of the unexpressed type IIC element DNA was seen.

D. Association with Other Repeats Appears to be a General Property of Many Type II IAP Elements.

Genomic DNA was cut with enzymes which cut inside the type II IAP elements only once, leaving the element associated with either 5' or 3' flanking DNA sequences. Discrete, prominent fragments larger than the elements themselves were seen in both types of digests probed with AIIins, suggesting that many of these IAP elements are associated with other repeats to form larger units. Such repeats occur in the 5' as well as 3' flanking regions.

E. Type II IAP Elements are on a Limited Number of Mouse Chromosomes.

In situ hybridization of AIIins to mouse metaphase chromosomes has revealed that these sequences are clustered and are not widely distibuted. We have initiated experiments using Southern blotting of DNAs from mouse x Chinese hamster somatic cell hybrids containing small numbers of mouse chromosomes to map these sequences to specific chromosomes. Association of multiple different types of repeats and extensive methylation of sequences that are parts of repeated DNA clusters have been noted. The clusters of type II sequences may be markers for such a specific region of DNA which is restricted for transcription.

F. Antisense Type II Transcripts are Expressed in Normal Cells.

AIIins has been inserted into vectors containing T3 and T7 RNA polymerase promoters to efficiently generate large amounts of RNAs complementary to either strand. Clones containing three AIIins in tandem were selected to increase strength of signal for use as probes. As expected, the antisense RNA probe detected 4.7 and 4 Kb type IIA and IIB IAP poly(A) RNAs in myeloma

cells. The sense RNA probe detected antisense poly(A) RNA in a variety of normal tissues which do not express type II RNA but do express type I poly(A) RNA. The antisense RNAs in mouse tissues had discrete sizes of 4.6 and 2 Kb; Syrian hamster cells contained a 5.2 Kb RNA in addition to these. Earlier work indicates antisense type I IAP transcripts also exist. It is possible that the antisense RNA plays some role in control of IAP sense RNA expression.

Significance for Cancer Research:

Type II IAP elements are expressed and transposed in transformed cells. They are a potential source of genetic variation in the mouse and could play a role in tumor development and/or progression.

Proposed Course of Research:

A cDNA library will be made from size selected poly(A) RNA from MOPC-315 myeloma cells to isolate clones representing actively transcribed type II IAP elements. Clones which are positive by hybridization with AIIins probe will be tested for protein coding capacity. The type IIB elements contain the coding regions for reverse transcriptase and endonuclease which are highly conserved among the IAP elements and extracellular retroviruses from other species. A polypeptide from this region will be synthesized and used to produce antibodies which can be used to identify clones expressing these proteins.

Publications:

Lueders, K.K. and Mietz, J.A.: Structural analysis of type II variants within the mouse intracisternal A-particle family. Nucl. Acids Res. 14: 1495-1510, 1986.

Kuff, E.L., Fewell. J.W., Lueders, K.K., DiPaolo, J.A., Amsbaugh, S.C., and Popescu, N.C.: Chromosome distribution of intracisternal A-particle sequences in the Syrian hamster and mouse. Chromosoma 93: 213-219, 1986.

Feenstra, A., Fewell, J., Lueders, K.K., and Kuff, E.L.: In vitro methylation inhibits the promoter activity of a cloned intracisternal A-particle LTR. Nucl. Acids Res. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05265-04 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

P. Wagner	Guest Researcher	LB	NCI
J. Schmidt	Technician	LB	NCI
N.-D. Vu	Staff Fellow	LB	NCI

COOPERATING UNITS *(if any)*
None

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

B

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

Interactions between cytoplasmic myosin and actin are responsible for a variety of cellular motile activities. As in muscle contraction, hydrolysis of ATP by myosin provides the required energy. While the actin-activated ATPases of vertebrate smooth muscle and nonmuscle myosins are regulated by phosphorylation of their 20,000-Da light chains, the mechanism of this regulation varies from one type of cell to another. Unphosphorylated gizzard myosin appears to be inactive, and phosphorylation causes a large increase in its actin-activated ATPase. This myosin has frequently been used as a model for all vertebrate smooth and nonmuscle myosins. However, we have found that phosphorylation regulates the actin-activated ATPase of calf thymus cytoplasmic myosin not by increasing the maximum rate of ATP hydrolysis but rather by increasing its affinity for actin. At low actin concentrations, phosphorylation stimulates the ATPase activity of thymus myosin, but at high actin concentrations or in the presence of tropomyosin, unphosphorylated and phosphorylated thymus myosin have comparable activities. We have subsequently found that the mechanism of activation of a mammalian smooth muscle myosin isolated from calf aorta is more like that of thymus myosin than that of gizzard myosin. This mammalian smooth muscle myosin has significant actin-activated ATPase activity in the absence of light chain phosphorylation. We have used skeletal muscle myofibrils from which the myosin has been extracted to demonstrate that unphosphorylated thymus and aorta myosins, but not unphosphorylated gizzard myosin, can cause contraction. The ability of unphosphorylated aorta myosin to cause contraction is consistent with the maintenance of tension by mammalian smooth muscles when light chain phosphorylation has returned to resting levels. These results also suggest that mammalian cells contain some mechanism in addition to light chain phosphorylation for regulating actomyosin based motility.

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.

Methods Employed:

Standard protein isolation techniques are used to prepare actin, myosin, myosin light chain kinase, and other cytoskeletal proteins from various types of muscles and from thymus and brain. Characterizations of their interactions are done using enzymatic assays, fluorescence spectroscopy, ultracentrifugation, and light and electron microscopy.

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 20,000 and 17,000. The 20,000-Da light chains are phosphorylated by Ca^{2+}-calmodulin dependent kinases and dephosphorylated by Ca^{2+} insensitive phosphatases. This phosphorylation is generally believed to be the primary mechanism for regulating the actin-activated ATPases of these myosins and, consequently, smooth muscle contraction and nonmuscle contractility. The most detailed information so far available on the mechanism of this regulation comes from experiments with turkey gizzard smooth muscle myosin. However, as we reported last year, the mechanism of activation of calf thymus cytoplasmic myosin is very different from that of gizzard myosin. Unphosphorylated gizzard myosin appears to be inactive, and phosphorylation causes a large increase in its actin-activated ATPase activity. Both heads of gizzard myosin must be phosphorylated before either head is activated. In contrast, phosphorylation and the resulting stimulation of one head of thymus myosin is independent of the phosphorylation of the other head. Furthermore, phosphorylation regulates the actin-activated ATPase of thymus myosin not by increasing the maximum rate of ATP hydrolysis but rather by increasing its affinity for actin. At high actin concentrations or in the presence of tropomyosin, unphosphorylated and phosphorylated thymus myosin have comparable ATPase activities. We have now examined the phosphorylation of calf aorta myosin to determine whether the activation of this mammalian smooth muscle myosin is more like that of thymus or gizzard myosin.

When electrophoresed under nondenaturing conditions myosins with two phosphorylated light chains migrate more quickly than myosin with one phosphorylated light chain. This technique was used to determine the order of phosphorylation of aorta myosin. Both monomeric and filamentous aorta myosin appeared to be phosphorylated randomly. Thymus myosin is also phosphorylated randomly, but there is disagreement as to the order of phosphorylation of gizzard myosin.

The dependence of the actin-activated ATPase of calf aorta myosin on both the level of light chain phosphorylation and on the actin concentration was deter-

mined. Under conditions where both unphosphorylated phosphorylated aorta myosins are mostly filamentous, the maximum rate of the actin-activated ATPase of the unphosphorylated myosin is one-half that of the phosphorylated myosin. In the presence of tropomyosin, phosphorylation also caused a 10-fold decrease in K_{app}, the concentration of actin required to achieve 1/2 V_{max}. In the presence of low concentration of actin and tropomyosin, a linear relationship was obtained between the fraction of light chain phosphorylated and stimulation of the actin-activated ATPase. The actin-activated ATPase of thymus myosin also increases linearly with phosphorylation, but the actin-activated ATPase activity of gizzard myosin increases with the square of the fraction of light chain phosphorylated. Since aorta myosin is phosphorylated randomly, this linear relationship between phosphorylation and ATPase activity suggests that phosphorylation of one head of aorta myosin stimulates the actin-activated of that head independent of the phosphorylation of the second head. Thus, the activation of aorta myosin appears to be more like that of thymus myosin than that of gizzard myosin.

Washing skeletal muscle myofibrils with a high salt solution results in the extraction of myosin, but actin remains attached to the Z-lines. These "ghost" myofibrils do not contract in the presence of ATP, but ghosts reconstituted with skeletal myosin contract rapidly when ATP is added. Ghost myofibrils were reconstituted with unphosphorylated and phosphorylated turkey gizzard, calf aorta, and calf thymus myosins. While ghost myofibrils reconstituted with unphosphorylated gizzard myosin did not contract, those reconstituted with unphosphorylated thymus and aorta myosins did contract. All three phosphorylated myosins supported contraction. The contraction of ghost myofibrils by unphosphorylated calf thymus and aorta myosins demonstrate that these myosins can cause movement.

Significance to Biomedical Research and the Program of the Institute:

Cell movement, endocytosis, capping of cell surface receptors, cell division and a variety of other cellular activities are thought to rely on forces generated by the interactions of cytoplasmic actins and myosins. The actin-activated ATPase of gizzard myosin has been used as a general model for the interaction of vertebrate smooth muscle and nonmuscle myosins with actin. The experiments reported here indicate that the actin-activated ATPase activities of some mammalian cytoplasmic and smooth muscle myosins are regulated very differently than that of gizzard myosin. The ability of unphosphorylated aorta myosin to cause contraction provides a simple explanation for the maintenance of tension by mammalian smooth muscle when light chain phosphorylation has returned to resting levels. These results also indicate that mammalian cells contain some system in addition to light chain phosphorylation for regulating actomyosin based motility.

Proposed Course:

The major limitation of the contraction of ghost myofibrils to measure contractile activity is that it is impossible to measure rates of movement. A more quantitative assay is the movement of myosin coated beads along the orientated actin cables of Nitella. The rate of bead movement correlates with the actin-

activated ATPase activity of the myosin used to coat the bead. This assay will allow us to determine the rates at which thymus and aorta myosin can move. It can also be used to examine the effects of actin binding proteins on movement.

Although light chain phosphorylation causes an increase in the affinity of thymus myosin for actin, this change appears to be too small for phosphorylation to be an effective regulatory system. This result raises two questions. Is there another regulatory system for actomyosin based motility in these cells, and what is the precise role of light chain phosphorylation?

An obvious place to look for other regulatory factors is among the large number of actin binding proteins which have been isolated from nonmuscle cells. Rather than isolating several of these proteins and testing their effects on a reconstituted system, I propose to prepare intact thin filaments from thymus and to study their interactions with unphosphorylated and phosphorylated thymus myosins in the presence and absence of Ca^{2+}. If the thymus thin filaments have preserved their regulatory properties, the regulatory proteins will then be isolated and their effects both on actin-activated ATPase activity and on the movement of thymus myosin coated beads along the actin cables of Nitella determined.

Myosin light chain phosphorylation also affects the stability of filaments of cytoplasmic myosins. MgATP dissociates filaments of unphosphorylated cytoplasmic myosins to a much greater extent than those of the phosphorylated myosins. Thus, light chain phosphorylation may regulate the transient assembly of myosin filaments at different locations within the cell where they are required for a particular type of motion. However, the physiological significance of this depolymerization is uncertain. At physiological ionic strength, MgATP also depolymerizes unphosphorylated smooth muscle myosins, but in relaxed smooth muscles most of the unphosphorylated myosin is filamentous. The low myosin concentration in nonmuscle cells makes it difficult to detect myosin filaments by conventional microscopic techniques. However, an immunofluorescence procedure has recently been described for visualizing myosin filaments in Dictyostelium. While these filaments are larger than those of mammalian cytoplasmic myosins, the technique may be modified to allow us to determine whether unphosphorylated cytoplasmic myosins in mammalian cells are filamentous and to examine changes in myosin filament formation and distribution.

Publications:

Wagner, P.D., Vu, N., and George, J.N.: Random phosphorylation of the two heads of thymus myosin and the independent stimulation of their actin-activated ATPases. J. Biol. Chem. 260: 8084-8089, 1985.

Wagner, P.D., and George, J.N.: Phosphorylation of thymus myosin increases its apparent affinity for actin but not its maximum adenosine triphosphatase rate. Biochemistry 25: 913-918, 1986.

Wagner, P.D., and Vu, N.-D.: Regulation of the actin-activated ATPase of aorta smooth muscle myosin. J. Biol. Chem. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05266-04 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

Cary Queen	Research Chemist	LB	NCI
L.-T. Bich-Thuy	Visiting Fellow	LB	NCI
J. Stafford	Technician	LB	NCI (4 months)
S. Hu	Technician	LB	NCI (6 months)

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Macromolecular Interactions Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are studying the regulation of expression of the immunoglobulin gene family by attempting to answer two questions: (1) why do only cells of the B-lymphoid lineage synthesize immunoglobulins, (2) how do these cells transcribe only one or a few immunoglobulin genes, while leaving hundreds of other, similar immunoglobulin genes inactive? Our approach to these questions is to insert cloned immunoglobulin genes into plasmids in various configurations, to transfect the plasmids into various types of cells, and to determine whether the transfected gene is transcribed. We have shown that a kappa immunoglobulin gene is transcribed after transfection into antibody-producing myeloma cells but not in non-lymphoid 3T3 or L cells. Hence the different cell types are able to appropriately regulate the kappa gene even when not in its usual chromosomal environment. By deleting different parts of the cloned gene, we have shown that an enhancer element downstream of the promoter is necessary for its transcription in myeloma cells. We have also demonstrated that both the enhancer and the promoter are cell-type specific, that is, they function in lymphoid cells but not in non-lymphoid cells. Recently, we have observed that the kappa enhancer stimulates the kappa promoter 20-fold more than it stimulates other promoters. This synergism between the kappa enhancer and promoter suggests the existence of a protein that binds to both regulatory elements.

GPO 914-918

Project Description

Objectives:

To study the regulation of the immunoglobulin gene family. In particular, to learn about the DNA sequences and protein factors that activate expression of immunoglobulin genes.

Methods Employed:

The project involves in vitro modifications of a cloned immunoglobulin gene. The modified genes are transfected into cultured cells of various types by the DEAE-dextran method. RNA is extracted from the cells and assayed by the S1 nuclease method to determine whether the immunoglobulin gene is transcribed.

Major Findings:

Regulation of the Immunoglobulin Gene Family

Each mammalian cell contains several hundred immunoglobulin genes, which encode the proteins that constitute antibodies. Only cells of the B-lymphocyte class (B-lymphoid cells) express these genes, that is, transcribe them into RNA and translate the RNA into immunoglobulin proteins. Moreover, out of the large repertoire of immunoglobulin genes, each antibody-producing cell generally expresses only one light chain gene and one heavy chain gene. It is of fundamental importance to the study of the immune system, and to the study of gene regulation during development, to learn why only certain cells express immunoglobulin genes, and how these cells are able to transcribe only two of them while leaving many similar genes inactive.

Our approach to this problem is to focus on one particular immunoglobulin gene, cloned from the mouse, that synthesizes a kappa light chain. This gene has been inserted on a plasmid that also contains a large part of the animal virus polyoma, allowing it to replicate in mouse cells. For one set of experiments, a second gene was inserted on the same plasmid. This gene was unrelated to the immunoglobulin system and, therefore, should be expressed at equal levels in B-lymphoid and non-lymphoid cells. The final plasmid, designated pLX31, was transfected into two standard non-lymphoid lines of mouse cells, 3T3 and L, and one antibody-secreting lymphoid line, MPC 11. As determined by an S1 nuclease assay of extracted RNA, the second gene was transcribed at approximately equal levels in all three lines of cells, as expected. This proves that the plasmid pLX31 was able to penetrate all of the cell lines in a transcribable state. However, the kappa immunoglobulin gene on the plasmid was only transcribed in the lymphoid MPC 11 cells, with no detectable transcription in the non-lymphoid 3T3 and L cells. Most recently, the same plasmid has been transfected into T-lymphoid cells, with results intermediate between the myeloma cells and non-lymphoid cells.

Two conclusions relevant to gene regulation during development can be inferred from this cell-type specific expression of a transfected kappa gene. First, since the DNA template is in the form of an unintegrated plasmid, the level of

kappa gene activity is not controlled by chromosomal location or large-scale chromosome structure. Second, since the kappa gene was transfected into terminally differentiated cells, activation or inactivation of this gene does not depend on modifications made to it during the process of cell development. Rather, the already differentiated cells contain all the information needed to appropriately regulate the expression of new copies of the kappa gene.

In previous experiments, we have shown that transcription of an immunoglobulin gene requires two DNA elements: (1) the promoter itself and (2) a downstream regulatory element called an enhancer that stimulates transcription from the promoter. In light of the results described above, the question arises whether it is the promoter or the enhancer that confers cell-type specificity on the immunoglobulin gene. Our recent experiments show that in fact both the promoter and enhancer are cell-type specific, i.e., function only in lymphoid cells. To show this, we first placed the immunoglobulin enhancer on a plasmid next to a viral promoter that can function in any cell type. We observed that the enhancer stimulated transcription from the promoter only when the plasmid was transfected into lymphoid cells, indicating that the enhancer is cell-type specific. Conversely, we placed an enhancer that can function in any cell type on a plasmid next to the immunoglobulin promoter. After transfection of the plasmid, the promoter functioned to initiate transcription in lymphoid but not non-lymphoid cells. As the enhancer works in either cell type, this shows that the promoter itself only functions in lymphoid cells.

Most recently, we have investigated the question of how κ light chain genes synthesize an equimolar amount of RNA with immunoglobulin heavy chain genes, even though the κ enhancer is only 10% as strong as the heavy chain enhancer. In the course of this work, we observed that the κ enhancer stimulates the κ promoter about 20-fold more than it stimulates other promoters. This is apparently the first time that a particular synergism between an enhancer and its "own" promoter has been noted. The synergism between the κ enhancer and promoter might be due to the action of a protein that specifically binds to both regulatory elements. We have also found that a κ promoter is about 10-fold stronger than a heavy chain promoter. Hence, it appears that a stronger κ promoter almost exactly compensates for a weaker κ enhancer.

Significance to Biomedical Research and the Program of the Institute:

The immunoglobulin gene family produces the proteins that constitute antibodies, a crucial aspect of the body's defense against infectious diseases and probably cancer. Understanding how these genes are regulated, especially how they are activated, is therefore of great potential utility in controlling disease processes.

Future Course of Research:

We will attempt to locate more precisely, at the nucleotide level, the DNA sequences involved in regulating expression of immunoglobulin genes. We will also use the differential expression of transfected immunoglobulin genes in lymphoid and non-lymphoid cells to develop an assay for the protein factors that activate transcription of immunoglobulin genes.

Publications:

Queen, C., Foster, J., Stauber, C., and Stafford, J.: Cell-type specific regulation of a κ immunoglobulin gene by promoter and enhancer elements. Immunological Reviews 89: 49, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05267-02 LB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

M. Yarmolinsky	Chief, Developmental Biochemistry and Genetics Section, LB, NCI		
D. Chattoraj	Research Chemist	LB	NCI
E. Hanson	Visiting Fellow	LB	NCI
S. Pal	Visiting Fellow	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:
4.0	4.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

We seek to understand replication and partition mechanisms that account for the stable inheritance of unit-copy bacterial plasmids. The P1 plasmid prophage chosen for study is representative of a class of genetic elements characterized by the presence of iterated DNA sequences at the origin of plasmid replication and in a replication control region. It is also characterized by a specialized partition apparatus. In the period of this report our laboratory has provided conclusive evidence for the existence of a novel biological control mechanism in which the control region acts in trans without mediation by messenger RNA. Means of testing the validity of alternative schemes by which this control mechanism works have been devised. We are also interested in the roles played by alternative origins in P1. From P1 phages that cannot replicate from their normal dnaA-dependent origin, insertion mutants which fail to replicate from an alternative dnaA-independent replicon have been obtained. Surprisingly the phage is still proficient for replication, indicating the presence of yet another origin. Preparatory to the isolation of bacteria with altered capacity for plasmid partitioning, λ-miniP1 hybrids have been constructed bearing markers that permit selection, counterselection and visual discrimination of mutants.

GPO 914-918

Project Description

Objectives:

We aim to elucidate mechanisms that (1) achieve precise control of the initiation of DNA replication and (2) assure the stable inheritance of replicons by appropriately partitioning them to daughter cells at cell division. With regard to the first aim, we seek to resolve a major paradox: the existence of apparently antagonistic controls on a gene that determines a protein essential for bacterial plasmid replication. These controls involve a novel role of DNA, namely an action in trans that is not mediated by messenger RNA. We also seek to understand the role of multiple origins in a plasmid by examining the effect of mutations that block the major plasmid origin of P1 from functioning. With regard to the second aim, we have constructed strains for the eventual isolation of host mutants altered in plasmid maintenance.

Methods Employed:

Our analyses of gene function depend upon *in vitro* methods of genetic engineering including the construction of gene fusions and *in vivo* methods of construction such as selection of integratively suppressed bacterial mutants by prophage displacement. DNA hybridization analysis was used to measure plasmid copy number and to verify constructions. Radioimmunoassays were used to measure the low levels of the P1-specific replication protein, RepA.

Major Findings:

Introduction

Plasmids P1 and F are representatives of a class of bacterial plasmids that are maintained with great stability at copy numbers approaching one per baby cell. A small portion of the plasmid genome serves to achieve stable plasmid maintenance. The region can be divided into functionally separate and physically separable *rep* (replication) and *par* (partition) elements. In P1 the 1.5 kb *rep* region consists of an origin (*oriR*), the gene (*repA*) for essential replication protein and a downstream region (*incA*) that exerts replication control. The organization is similar in F. The P1 RepA protein binds to a set of five 19 bp repeats within *oriR* which also includes the operator site of the *repA* promoter. The protein also binds to the 9 repeats that are present in *incA*. The RepA protein strongly represses transcription of the *repA* gene so that relatively few molecules of the protein are present per cell. The *incA* region appears to exert control over replication by sequestering RepA. Subsets of the *incA* repeats also exert replication control. Simultaneous autoregulation and sequestration would appear to be incompatible modes of controlling RepA concentration, yet both controls appear to operate under physiological conditions. One of the aims of our research is to resolve this paradox. By assuming a marked hierarchy of RepA affinities for binding sites (*rep* o/p >> *incA* > *ori*) it is possible to construct a model in which the binding sites fill up sequentially and cause the *ori* to fire periodically. Such a model, first proposed by Chattoraj, Abeles & Yarmolinsky, has been shown by David Womble to imply that the replication frequency should be exceedingly sensitive to changes in RepA concentration. An

alternative model, proposed by Trawick and Kline, supposes that RepA protein exists in two forms, of which one is an autorepressor, the other enters into ori/incA competition. The conversion of one form (A) to the other (B) is irreversible. Thereby form A is blind to the extent to which B is used up or sequestered. According to this model, the forms of RepA should be distinguishable by their binding properties.

The par region of P1 consists of a centromere-like element that appears capable of binding one or both of the par proteins which the phage specifies. Other proteins involved in partitioning are presumably host-determined.

Results

A. Deletion of incA from the basic oriR replicon of P1 and the role of incA.

The entire set of nine 19 bp iterons of incA has been deleted from the basic oriR replicon of P1 by means of standard recombinant DNA techniques. The deleted construct not only retained its capacity to replicate, but exhibited an eight-fold increase in copy number. This result dispels any residual doubt about the dispensability of the control region. A disproportionately large regulatory activity was retained when just one repeat was present. The single repeat unit was shown to be effective even when DNA was inserted between this remaining repeat and the normally adjacent repA gene. This result reveals that the repeat units, although capable of acting singly, do not act in simple additive fashion. Alternatively, minor differences between them account for large differences in biological effects. Cooperative activity of the repeats, if it exists, is not essential for biological effectiveness. The essential feature of the repeats was shown to be their capacity to bind RepA protein. By both in vivo and in vitro assays the single repeat unit was found to bind RepA. When part of this repeat was deleted and its binding potential lost, biological activity in copy number control was lost too. These studies establish that the role of incA in copy number control is uniquely by sequestering RepA protein.

B. In vivo protein-DNA binding assayed by competition for an operator

The autoregulatory role of RepA, previously demonstrated in this laboratory, was exploited to develop a simple enzymatic assay for the efficiency with which repeated DNA sequences (iterons) sequester RepA protein. In this assay competition for RepA protein is between iterons introduced on a plasmid and the repA operator/promoter which is fused to a lacZ gene. The RepA protein is furnished at a constant but low rate from a plasmid so that modest sequestration is adequate to induce appreciable β-galactosidase synthesis. Plasmid copy numbers in this and other experiments were measured by a blot DNA hybridization method that is accurate to within ±20% despite a 5000-fold excess of extraneous DNA. These assays permit the RepA-binding proficiency of different sets of iterons or individual iterons to be compared. Preliminary results show that incA iterons appear no less effective than ori iterons in binding the form of RepA that binds to the operator of repA. These results directly contradict the Trawick and Kline proposal

described in the Introduction and have led to the formulation of a novel alternative explanation of the regulatory circuitry, involving a DNA fold-back.

C. Dependence of P1 on dnaA

It was concluded that dnaA is necessary for oriR replication in the annual report of last year. This work has continued and a phase of it completed. A surprising capacity of wild type P1 to lysogenize a dnaA null mutant or of a P1 repA mutant to lysogenize a wild type E. coli was shown to be due to integration of the P1, apparently mediated by an IS1 element that is a component of the P1 genome. This result explains how P1 can lysogenize without autonomous replication, but raises other questions about the inefficiency of integration under normal conditions.

P1 can be shown to possess dnaA independent replicon(s), because a P1 prophage is able to suppress the replication defect associated with a dnaA null mutation in E. coli. Insertion mutants of P1 with altered ability to suppress bacterial dnaA ts mutants have been isolated. A Tn5 insertion that results in loss of function of the dnaA-independent replicon of P1 appears still capable of forming a normal plaque. The insertion is in the vicinity of the oriL replicon. P1 repA$^-$ was also found to make normal plaques. These results bear upon the relationship of replication from oriR and oriL to lytic replication of the phage.

D. Host functions in plasmid maintenance: selection of bacterial mutants

In order to select host mutants in which plasmid stability is decreased, we have chosen a simple system, a plasmid that contains no backup mechanisms such as alternative replicons or means of delaying cell division in cells that would otherwise lose plasmid. The simplest P1 plasmid of reasonable stability is stabilized only a few fold by its partitioning genes in a rec$^+$ host, appreciably more in a recA host. In either case the selection of mutants affected in partitioning must require recycling. The plasmids currently used for this purpose are lambda-mini-P1 hybrids. Lambda allows the plasmids to be easily introduced by infection and furnishes the counter-selective marker (cI857). Phages with drug resistance markers have been constructed and one of these was also furnished with the E. coli lacI gene to allow for a sensitive identification of plasmidless cells in a lacI$^-$ background. To prevent reinfection of plasmidless cells at any time during the selection procedure the phages were rendered conditionally defective with amber mutations in the lambda head genes W and E.

From heavily mutagenized E. coli two strains showing decreased miniP1 stability were isolated. We are encouraged that it has been possible to obtain strains of the desired phenotype by this method and are currently optimizing the method prior to isolating and classifying additional mutants.

Significance to Biomedical Research and the Program of the Institute:

The present effort advances knowledge of how DNA synthesis is controlled and is aimed at advancing knowledge of critical processes in cell division using a

simple, well-studied biological system that is amenable to thorough analysis. A possible application of our knowledge of P1 replication control to understanding replication control in Epstein-Barr Virus (based on structural similarities between the bacterial and animal virus) reinforces our conviction that the research we describe is likely to affect cancer biology in ways that are no less important for being unpredictable.

Future Course of Research:

We will attempt to define the separate functional domains of the RepA protein of P1 involved in initiating replication, inhibiting replication (when in excess), binding to repeat sequences (including synthetic oligonucleotides) and auto-regulation. We will use this information to understand the regulatory circuitry that maintains the P1 copy number at its constant low value.

We will continue our analysis of alternative replicons in P1 to access their importance and their modes of control.

A major effort will be devoted to obtaining and characterizing host mutants that alter the efficiency of plasmid maintenance, particularly those affected in the process of plasmid partitioning.

Publications:

Hansen, E.B., and Yarmolinsky, M.: Host participation in plasmid maintenance: dependence upon dnaA of replicons derived from P1 and F. Proc. Natl. Acad. Sci. USA (in press).

Pal, S.K., and Chattoraj, D.K.: RepA is rate limiting for P1 plasmid replication. In Kelly, T., and McMacken, R., (Eds.): Mechanisms of DNA Replication and Recombination. UCLA Symposium on Molecular and Cellular Biology, New Series, Vol. 47. New York, Alan R. Liss, Inc. (in press).

SUMMARY STATEMENT

ANNUAL REPORT
LABORATORY OF CELLULAR ONCOLOGY
DCBD, NCI
October 1, 1985 through September 30, 1986

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 and papillomaviruses. We have studied the ras containing Harvey murine sarcoma virus (Ha-MuSV). A series of inframe mutants of the Ha-MuSV ras transforming gene have been used to correlate the capacity of this gene to transform established mouse cells with the known biochemical and topological features of ras proteins. The results have defined multiple regions required for transforming activity and nucleotide binding and have also led to the identification of a new class of mutants whose lesions appear to define a region of ras that may interact with its cellular target. In vivo studies of Ha-MuSV have shown that deletion of a segment of non-ras viral sequences changes the type of tumor induced by the virus. This change in target cell specificity appears to be associated with the presence of an enhancer element located in this region. In papillomavirus studies, the bovine papillomavirus genome has been analyzed. We have determined that the second transforming gene of the Bovine papillomavirus is encoded by the E5 open reading frame. It has also been noted that the E1 gene normally inhibits the efficiency of cell transformation by the viral DNA. In addition, a peptide encoded by the E2 open reading frame has been shown directly to bind to several sites within the upstream regulatory region of the BPV genome and the HPV16 genome. Since the E2 gene has previously been shown to encode a trans-acting factor that enhances the transcription directed from this region, the results suggest that this activation is mediated via the direct interaction between the E2 protein and the viral regulatory region.

Regulation of retroviral replication and cellular oncogene expressions

The long range purpose of this project is to investigate the roles of cellular oncogenes and retroviruses in neoplasia and to use viral mutants for elucidating regulatory mechanisms of gene expression associated with cell differentiation and oncogenesis. The possible activation of cellular oncogenes in the spontaneous Hodgkins-like reticulum cell neoplasm of the SJL/J mouse has been analyzed. Preliminary results suggest that in 5 out of 12 tumors studied an endogenous retroviral genome has integrated within a few kilobases of the c-abl proto-oncogene locus. These results suggest that the development of this

neoplasm may be associated with the activation of c-abl. In a model system that employed an anchorage dependent mink cell line non-productively infected with Moloney sarcoma virus (which contains the v-mos oncogene), superinfection with a novel dualtropic mouse retrovirus induced anchorage independent cell growth that correlated with a marked amplification and increased expression of the v-mos oncogene.

Genetic mechanism of carcinogenesis and biological modifiers as defense mechanism

The major goals of this project are to elucidate the molecular genetics of neoplastic transformation of normal tissues and the purification and function of biological modifiers that may be important to host defense. A transforming gene has been detected in a human hepatocarcinoma cell line. The clone displays a low constitutive level of transforming activity on NIH 3T3 cells. Treatment of the DNA with AFB-1-epoxide results in a more than 300-fold increase in transforming activity. The sequence represented in the clone appears to be amplified significantly in many hepatomas. Nucleotide sequence analysis has indicated the presence of an open reading frame that could encode a peptide of 155 amino acids. Additional studies are required to determine if this gene is related to previously described oncogenes. In biological modifier studies, a lymphokine called cytotoxic cell differentiation factor (CCDF) has been studied functionally and purified from unstimulated murine peritoneal macrophages. CDCC is required in conjunction with IL-2 to form lymphokine-induced cytotoxic cells (LICC), which are thought to represent an important host defense mechanism against neoplastic cells.

Biological studies of various normal, virus-infected, and malignant cells

The primary purpose of this project is to study some of the pertinent factors which influence cell differentiation and malignant transformation. A major current emphasis is to define more precisely the mechanisms by which interferon (IFN) acts on cells. The test system used is the virus-negative methyl-cholanthrene induced BALB/c sarcoma, Meth A, which can be passed both in vivo and in vitro. In this system, comparison of the response in cell culture with that in the intact mouse makes it possible to analyze the direct action of IFN on Meth A cells separately from the host defenses. Cells treated in vitro were analyzed for the anti-cellular effects in vitro and immune reactivity in vivo. At the concentrations tested, IFN was cytostatic, but not cytotoxic. Meth A cells treated with alpha-beta IFN were rejected more efficiently than those treated with gamma IFN. The ability of immunodeficient mice to support the growth of IFN treated Meth A cells was compared with their growth properties in immunocompetent mice. Immunodeficient mice included nude mice as well as mice rendered immunocompromised by treatment with either cyclophosphamide or cyclosporin A. In each instance, the IFN treated cells grew much better in the immunodeficient animals. These experiments confirm the necessity of functional T cells in order for IFN to exert its antitumor effect. The results obtained with IFN treatment of Meth A suggest that the major effect of IFN on chemically induced sarcomas is mediated through the host immune response, rather than by its anti-cellular activities.

Biology of fps/fes oncogenes

This project aims at elucidating the biological function(s) of the normal human c-fps/fes proto-oncogene, and to understand the molecular and cellular basis of its oncogenic potential. We have demonstrated that a cloned human genomic DNA fragment containing c-fps/fes-homologous sequences contains the entire coding sequence of the human c-fps/fes-encoded protein. Our results confirmed that this protein is the 92 Kda cellular tyrosine kinase that we have previously identified as the gene product of human c-fps/fes. Using a retroviral shuttle vector system, we have been able to recover an intron-less copy of human c-fps/fes DNA. We have also identified and begun characterization of a new cellular tyrosine kinase present in all vertebrate cells that is immunologically related to the c-fps/fes product, but is encoded by a different gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03663-10 LCO

PERIOD COVERED

October 1, 1985 through September 30, 1986

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)*

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	J. T. Schiller	Senior Staff Fellow	LCO NCI
	W. S. Sawchuk	Medical Staff Fellow	LCO NCI
	K. H. Vousden	Visiting Fellow	LCO NCI
	T. J. Velu	Guest Researcher	LCO NCI
	N. L. Hubbert	Microbiologist	LCO NCI
	A. G. Papageorge	Microbiologist	LCO NCI

COOPERATING UNITS *(if any)*

University Microbiology Institute, Copenhagen, Denmark, Dr. B. Willumsen
The Roche Institute, Nutley, NJ, Drs. H.-F. Kung and D. Stacey
Hopital Cochin, Paris, France, Dr. P. Tambourin

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
9.00	6.00	3.00

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.)*

An inframe mutational analysis of the v-rasH oncogene of Harvey murine sarcoma virus has indicated that most hydrophilic regions of the protein it encodes are dispensable for transformation by NIH 3T3 cells. One of these regions, which probably represents an area of the protein that interacts directly with its cellular target (S), is required for biological activity. In Harvey murine sarcoma virus, an enhancer region located outside the viral LTR has been identified. This enhancer element plays a major role in determining the pathology induced by the virus.

In studies of the Bovine papillomavirus genome, the second transforming gene of this virus has been identified as being encoded by the E5 open reading frame. The E1 open reading frame has been shown to inhibit the induction of cell transformation by BPV. A polypeptide encoded by the E2 open reading frame has been found to bind directly to multiple sites located within the upstream regulatory regions of the BPV and HPV16 genomes, suggesting that the enhancer activity of the E2 gene product on the viral genome is mediated via this direct interaction with viral DNA.

PHS 6040 (Rev. 1/84)

GPO 904-917

Other Professional Personnel:

W. C. Vass Biologist LCO NCI

Major Findings:

1. Structure function analysis of a ras oncogene. 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 ras counterpart at only 2 amino acids (12 and 59). In collaboration with the University Microbiology Institute, Denmark (B. Willumsen), and the Roche Institute (H. F. Kung and D. Stacey), we have studied the effect of inframe mutations (deletions, substitutions, and insertions) on the capacity of this oncogene to transform NIH 3T3 cells. The results have been correlated with the known biochemical and topological features of the ras proteins. The results have defined multiple regions required for transforming activity and nucleotide binding. In addition, they have led to the isolation of a new class of mutants whose lesions appear to define a region of ras that interacts with its putative cellular target. The hydropathic profile of the ras protein demonstrates six hydrophilic regions, suggesting that these regions are located on the exterior of the protein. Five of these six regions have been found to be dispensable for transformation, since extensive deletions within each of these failed to abolish the capacity of the gene to transform NIH 3T3 cells. However, minor substitution mutations in the fifth region (amino acids 31 to 42) destroyed the transforming activity of the gene, although the proteins encoded by these mutants displayed properties associated with oncogenic forms of ras: high GTP binding, low GTPase activity, and localization to the plasmid membrane. In contrast to these mutants, mutations in multiple hydrophobic regions of the protein markedly reduced or abolished the nucleotide binding of the protein and eliminated the transforming activity of the gene. The results suggest that amino acids 31 to 42, which are highly conserved among ras proteins, interact with the ras cellular target and that multiple regions elsewhere in the protein participate in guanine nucleotide binding.

2. Identification of VL30 sequences of Harvey sarcoma virus that contain an enhancer element. We have found that the VL30 sequences of Harvey murine sarcoma virus (Ha-MuSV) contain an enhancer that plays an essential role in determining the class of tumors induced by the virus.

The virus induces splenic erythroleukemia and fibrosarcomas in newborn NIH Swiss mice. The non-ras portion of the viral genome is derived from two classes of sequences: Moloney (Mo) MuLV (contributing the LTR plus 0.5 kb at the 3' end) and rat retrovirus-like VL30 sequences (contributing 3.5 kb flanking the v-ras-H gene) that serve no known function. We have found that when a segment of VL30 derived sequences located downstream from v-ras-H was deleted from the viral genome, the resulting virus did not induce the two tumors characteristic of the full-length viral genome, although it encoded a wild-type v-ras-H gene product. Instead, the virus reproducibly induced lymphoid leukemia.

Compared with the full-length viral genome, NIH 3T3 cells transformed either by the deleted viral DNA or by virus containing the deleted genome

formed smaller foci, grew less efficiently in agar, and contained less viral RNA and viral p21 protein. The sequences required for efficient NIH 3T3 transformation were genetically localized to a 0.2 kb region of the deleted VL30 sequences. DNA sequence analysis of this region revealed a core enhancer sequence (GAGGAAAG). This region was also shown to enhance CAT activity in NIH 3T3 cells in an orientation-independent manner when it was placed upstream from the promoter region of a pSV2-CAT vector that lacked an enhancer.

3. Identification of a second transforming gene in the Bovine papillomavirus genome. We have previously shown that the early region of the Bovine papillomavirus type 1 (BPV) genome contains two non-overlapping segments that can independently induce the morphological transformation of cultured cells. The transforming gene from the 5' end of the early region is encoded by the E6 open reading frame. The second transforming segment was previously localized to a 2.3 kb fragment from the 3' end of the early region. This fragment however contained four open reading frames. In order to determine which of these four open reading frames encodes a transforming gene, we introduced a series of insertion and deletion mutations into a subgenomic fragment of BPV DNA that contained this region activated by a retroviral long-terminal repeat. The results indicated that the E5 open reading frame encodes a gene that induces transformation of NIH 3T3 cells; the other three open reading frames do not appear to possess this biological activity. This open reading frame could encode a low molecular weight hydrophobic peptide.

4. Influence of the E1 gene of Bovine papillomavirus on cellular transformation. The E1 gene has previously been shown to be required for maintaining the episomal state of papillomavirus DNA in infected cells. Through the analysis of multiple BPV mutants, in which single linker insertions disrupted the E1 reading frame, we have found that expression of E1 inhibits BPV induced transformation. The E1 mutants induced foci of transformed cells much more rapidly than the wildtype BPV or mutants with insertions in several other regions of the genome. The E1 mutants also grew more efficiently in agar that did the wildtype. In co-transfection experiments, a vector constructed to express only the E1 open reading frame was able to inhibit the transforming activity of BPV E1 mutants, whereas an identical vector with an interrupted E1 gene did not. A mutant with its lesion in a region located upstream from the E1 open reading frame displayed a similar phenotype and complementation analysis indicated that it fell into the same complementation group as the E1 mutants. These results suggest that this region is also required for E1 function, presumably because it serves a regulatory role.

Whether the E1 gene product acts directly to inhibit the transformation process or modulates the expression of the transforming genes is currently under investigation.

5. The E2 open reading frame encodes a DNA binding protein. Previous studies have indicated that the E2 open reading frame encodes a trans-acting factor that can stimulate the transcription of BPV sequences located in the upstream regulatory region. It is not known whether this stimulation represents a direct or indirect effect of the E2 gene product. We have now obtained evidence that a polypeptide encoded by the E2 open reading frame can bind directly to this region of the BPV DNA. A bacterially encoded fusion protein

that is composed of the majority of the E2 open reading frame has been found to bind specifically to five different segments of the upstream regulatory region in the area that contains the enhancer element. This protein also binds specifically to the analogous region of the HPV16 DNA but fails to bind to SV40 DNA. Each of the binding regions contain a similar sequence motif that is contained within a 17 base pair stretch. Experiments are currently under way to determine the significance of this motif to the DNA binding and to relate this binding activity to the biology of BPV.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05550-17 LCO

PERIOD COVERED
October 1, 1985 through September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of retroviral replication and cellular oncogene expressions

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

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OTHER:	L.-C. Wang	Visiting Associate	LCO NCI
	C. Gao	Guest Researcher	LCO NCI

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LAB/BRANCH
Laboratory of Cellular Oncology

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INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.3	2.0	1.3

CHECK APPROPRIATE BOX(ES)

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

B

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

The long range purpose of this project is to investigate the roles of cellular oncogenes and retroviruses in neoplasia, and to gain insight in the mechanisms of gene regulation associated with cell differentiation and neoplasia.

The topics of current interest are: 1) The role of proto-oncogenes and endogenous provirus loci in the oncogenesis of spontaneous reticulum cell neoplasms (RCN) of SJL/J mice. We found a high incidence of rearrangements not only in the c-abl but also in the endogenous ecotropic provirus loci in these tumors. The results are consistent with the hypothesis that activation of c-abl by promotor/enhancer insertion involving endogenous ecotropic proviruses may play a role in some RCN oncogenesis in SJL/J mice. 2) The role of mos in the anchorage-independent (AI) growth and metastatic ability of S+L- mink cells superinfected with a novel dual-tropic retrovirus (E1BX-MuLV). A marked amplification and rearrangement of v-mos in the chromosomal DNA accompanied by an increased number of E1BX-MuLV proviruses integrated near v-mos loci, and an enhanced expression of v-mos mRNA were correlated with the AI phenotype, oncogenicity, and metastatic potential of S+L- mink cells superinfected with the E1BX-MuLV. 3) Oncogene amplification, rearrangement and expression in human embryonal carcinomas (EC), and chorio-carcinomas. Two cell lines of human EC, Tera-1 and Tera-2, showed an enhanced Ki-ras mRNA expression as well as an amplification and rearrangement of Ki-ras-2 gene. NIH/3T3 cells transfected with the Tera-2 DNA produced tumors in nude mice, and secondary and tertiary transfectants proved to be tumorigenic. Identification of the activated oncogene is in progress. 4) Monoclonal antibodies against HTLV-I. Among many hybridomas that were positive in the initial screening, two have been shown to produce monoclonal antibodies against 69 kd viral protein.

GPO 904-917

Major Findings:

1. The role of oncogenes and endogenous provirus loci in the oncogenesis of spontaneous reticulum cell neoplasms (RCN) of SJL/J mice. The SJL/J mouse exhibits a high incidence of spontaneous Hodgkin's-like reticulum cell neoplasm (RCN) at old ages (usually more than 8-10 months). We have investigated the status of ecotropic proviruses and various oncogenes in RCNs of SJL/J mice for their possible relationship to neoplasia. By blot-hybridization with v-mos, v-fos, v-sis, v-Ki-ras and N-ras as probes, we could not observe any significant rearrangement of oncogenes in DNAs extracted from RCNs or T-cell leukemia of SJL/J strain. However, it was found that, when a sequence representing 2/3 of v-abl on the 5' side was used as a probe, we could detect c-abl rearrangements in 7 out of 12 RCNs examined. The rearrangements were shown as a novel 19 kb and/or a 9 kb fragment in addition to the other fragments produced by HindIII cleavage. There were also new insertions of ecotropic provirus in 9 out of 12 RCNs tested. These new fragments were found either as a single 19 kb fragment, as 19 kb and 8.8 kb, or as 9.4 kb. The 19 kb fragments of c-abl was superposable on the 19 kb fragment of eco loci when the same filter blot was used. Thus 5 out of 12 RCNs exhibited an extra 19 kb fragment reacting with both v-abl and eco probes. In contrast, no c-abl rearrangement was detected in T-cell leukemias of SJL/J mice, although new insertions of ecotropic provirus loci were found. The 19 kb and 9 kb HindIII fragments observed in certain RCNs mentioned above did not hybridize with a probe representing 1/4 of v-abl on the 3' side. Since the transforming protein is encoded by the sequence representing 2/3 of v-abl on the 5' side, these extra fragments which hybridized with this portion of v-abl may include sequences coding for the transforming protein. These results are consistent with the hypothesis that activation of abl proto-oncogenes by promotor/enhancer insertion involving endogenous ecotropic proviruses may play a role in some RCN oncogenesis in SJL/J mice.

2. The role of mos in the anchorage-independent (AI) growth and metastatic potential of S+L-mink cells superinfected with a novel dual-tropic retrovirus (ElBX-MuLV). We have isolated new strains of dual-topic virus which exhibited a broad host range as well as a B-tropism. The S+L-mink cells superinfected with a strain of this virus (ElBX-MuLV) gave rise to transformed cells which readily detached spontaneously from the substratum. A clone of such AI cells was isolated and compared with a clone of infected transformed cells that were still showing anchorage-dependent (AD) growth. The AI clone grew in suspension, showed a high oncogenicity and ability to metastasize in nude mice, and a relatively high efficiency of colony formation in soft agar medium. In transfection assays, only the DNA from AI clone could transform NIH/3T3 cells. It was found that the v-mos was greatly amplified and rearranged in the AI clone, and moderately amplified and rearranged in the AD clone, as compared with the uninfected control cells. The amounts of mos-related mRNA expressed by these clones paralleled those of v-mos copies in the chromosomal DNA. However, the amounts of ElBX-MuLV-related mRNA were low, and no difference between the AI clone and the AD clone was observed, whereas the copies of ElBX-MuLV proviral sequence integrated were markedly increased in the AI clone as compared with those of the AD clone. So far, no other oncogene amplification has been detected in these cells. The results suggest that amplification and rearrangement of v-mos DNA with enhanced expression of its

mRNA appear to play an important role in the AI phenotype, oncogenicity, and metastatic ability of S+L-mink cells superinfected with E1BX-MuLV.

3. Oncogene amplification, rearrangement, and expression in human embryonal carcinomas and choriocarcinomas. Human embryonal carcinomas and choriocarcinomas are of special interest because the former has the potentiality to be induced to produce differentiated cells, and the latter is derived from trophoblasts which are of extraembryonic lineage. We have found that two human teratocarcinoma cell lines, Tera-1 and Tera-2, showed a Ki-ras-2 amplification and rearrangement as well as enhanced expression of this oncogene. The degree of amplification could not be explained simply by karyotype abnormalities. Chromosome 12, in which Ki-ras-2 is located, was found to be trisomic in most cells of Tera-2 but not Tera-1. However, some translocations and marker chromosomes are present in both cell lines. NIH/3T3 cells transfected with the chromosomal DNA of these cell lines showed a low frequency of transformed foci, and produced tumors in nude mice. The chromosomal DNA extracted from these tumors was used to generate secondary transfectants which again proved to be tumorigenic in nude mice. DNAs from the tumors produced by the secondary and tertiary transfectants are being characterized to identify the activated oncogene.

It is of interest to note that Tera-1 and Tera-2 cells as well as human trophoblast cells have been reported to produce type-C retroviruses, the identity of which is unclear. We found that the chromosomal DNA of Tera-2 cells contains a sequence cross-hybridizing with HTLV-I env gene probe under a moderate stringency of hybridization. A DNA probe representing the gag and env sequence of a human endogenous retrovirus, ERV3, was used to study its relationship with the proviral sequence in these embryonal carcinoma cells (in cooperation with M. Cohen, FCRF). No amplification or rearrangement of ERV3 proviral sequence in these cells has been found so far.

We have collected two more choriocarcinoma cell lines and also a number of tumor tissues including hydatidiform moles as well as human embryonal carcinomas. Their chromosomal DNAs are being tested for alterations in oncogenes and endogenous retroviral (ERV3) sequences. Their mRNA expression is being examined also, since we previously found an enhanced expression of c-fos mRNA in a line of choriocarcinoma. ERV3 mRNA expression is also of interest because of the report (M. Cohen et al.) that high levels of ERV3 env-containing transcripts are present in the normal placental tissues.

4. Characterization of monoclonal antibodies against HTLV-I antigens. Attempts have been made to produce and screen hybridomas for production of monoclonal antibodies against structural as well as nonstructural antigens of HTLV-I, which are important for viral pathogenesis, transmission, and gene regulation. Among 650 hybridoma clones, 9 showed reactivity against lysates of purified HTLV-I, 16 were positive against MT-2 (HTLV-I producer lymphocyte) antigens, and 52 reacted against C81-66-45 (HTLV-I transformed nonproducer lymphocyte) antigens. Two hybridomas were selected for mouse ascites production, and preliminary characterization by using Western blot-ELISA test indicated that the monoclonal antibodies can recognize a 69 kd protein from the purified HTLV-I virus lysate.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04834-10 LCO

PERIOD COVERED
October 1, 1985 through September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic mechanism of carcinogenesis and lymphokines in cellular defense

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

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OTHER:	J. V. Taub	Biolab Technician	LCO NCI
	R. Modali	Biologist	LCO NCI

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.50	1.00	1.50

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☐ (a1) Minors
☐ (a2) Interviews

B

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

The thrust of this study is to elucidate the genetic mechanisms of neoplastic transformation of normal tissues and the cellular immune defense mechanisms against neoplastic cells. Two experimental systems were used: 1) A human oncogene derived from a hepatocellular carcinoma established in culture (MAH); 2) IL-2, an autocrine of T-cells, and CCDF (cytotoxic cell differentiation factor), produced by macrophages for the generation of lymphokine-induced cytotoxic cells.

A systematic screening with DNA transfection assay, preferential AFB_1 binding and repeated molecular clonings, have been carried out and led to the successful isolation of a human hepatocellular carcinoma oncogene, hHC, which transforms NIH 3T3 cells at very low efficiency. Its transformation capability was, however, enhanced by more than 300 fold upon AFB_1 activation. One hHC clone, carried in Puc 8, is 3.1 kb and shares homology with human c-*fes*, c-*raf* and k-*ras*. We have established a restriction map of hHC and resolved the nucleotide sequence of the entire gene. By using a modified Maxam/Gilbert sequencing technique, the deoxyguanine targets in the hHC DNA sequence under controlled binding conditions with AFB_1 have been identified. This yields some relevant information regarding the nucleotide sequence specificity influencing AFB_1 activation of hHC. A cellular homolog of hHC, nhl, has been isolated from normal liver DNA. Restriction mapping of nhl suggests that, whereas homology between hHC and nhl is evident in the termini, recombination and rearrangement in the central portion of nhl has probably occurred to account for the structure of hHC.

After extensive purification, the function and relationship of two oncogene products, IL-2 and CCDF, have been analyzed. In the absence of antigenic or mitogenic stimulation, IL-2 provides the first signal to activate the cytotoxic precursors and prepares these cells to differentiate into cytotoxic effectors in the absolute presence of CCDF. The synergistic action of IL-2 and CCDF is found critical for the generation of lymphokine-induced cytotoxicity, a cellular process considered pivotal in cellular immune defense mechanisms against tumor cells.

Major Findings:

1. hHC human hepatocellular carcinoma oncogene: its homology with other known proto-oncogenes and oncogenes and its distribution among normal, neoplastic and pathological livers and other tissues. Human transforming DNA sequences related to the known oncogene families have been detected in various human tumors. We have isolated a human oncogene from a hepatocellular carcinoma cell line, MAH, established from an African patient (Mahlavu). MAH had no hepatitis B marker on the cell surface. In this on-going investigation we have identified the presence of inserts of the hepatitis B subgenomic sequences in MAH genome (see section 3 below). Our findings established that high molecular weight (HMW) hepatocellular carcinoma DNA failed to mediate cell transformation in NIH/3T3 cells (1-2 ffu/ 2-5 mg DNA). However, upon binding with AFB_1-epoxide MAH HMW DNA showed increased cell transformation efficiency (4-6 ffu/200 femtomole [^{3}H]AFB_1/10 ug DNA). AFB_1-epoxide bound DNAs from NIH/3T3 DNA, E. coli, normal human liver DNA, and c-K-ras DNA all failed to transform NIH/3T3 cells in spite of successful AFB_1 binding. A systematic approach using [^{3}H]AFB_1-epoxide binding for effective screening, hybridization against human [^{32}P]-Alu-sequence, and transfection assay on NIH/3T3 cells, was carried out to identify the MAH transforming DNA. With repeated clonings and screenings several clones carrying a Hind III-Hind III genomic DNA fragment, inserted in Puc 8, were obtained. One such clone, PM-1, carrying a 3.1 kb human hepatocellular carcinoma, hHC, DNA, showed capability of transforming NIH/3T3 cells without activation by AFB_1 although at very low efficiency (8-10 ffu/5-10 ug PM-1 DNA). Upon controlled activation with AFB_1-epoxide, PM-1 DNA showed more than 300 fold increase in cell transformation capability (17 ffu/24 femtomole [^{3}H]AFB_1/100 ng PM-1 DNA (see section 5).

We have examinined the distribution of hHC sequence in numerous human normal cell genomic DNA and either neoplastic or pathological liver cell DNAs. These included genomic DNAs isolated and purified from normal human liver, human skin (biopsy), WI-38 human fibroblast cell line, and 21 hepatomas (two Chinese and 19 Koreans), two hepatocellular carcinoma cell lines (MAH and PLC-Jane Alexander), Reye's syndrome, and three human T cell leukemia (HUT 78, 102 and HL60). Strong hybridization was observed between [^{32}P]hHC DNA with all the hepatoma DNAs, HL60 DNA and Reye's syndrome DNA, but only weak hybridization was observed with DNAs prepared from normal liver, WI 38 and skin. By slot-blot hybridization with progressive dilutions of the DNA samples against [^{32}P]hHC probe, we found that hHC sequence was greatly amplified among the 23 hepatoma DNAs, varying from 200 to 640 copies, 180 copies in HL60 leukemia DNA, and only 1-3 copies in normal liver DNA.

The localization of hHC sequence in the hepatoma DNAs was then examined by Southern blot hybridization with endonuclease restricted hepatoma DNAs against [^{32}P]hHC probe. Two discrete HindIII fragments, 6.8 kb and 7.1 kb, beside a high molecular weight DNA (>25 kb) were identified in the hepatoma DNA prepared from a Chinese patient (380 copies of hHC homologous sequence/genome). Currently these DNA fragments were molecularly cloned for further study.

Homology analysis was also carried out to identify hHC sequence (PM-1 clone) with numerous known oncogenes such as: (1) the ras family (Harvey ras: v-Hl and c-H-ras; Kirsten ras: KBE-2, HiHi-3, c-kras-1; and n-ras), (2) human

c-myb and c-myc, (3) human c-raf, (4) human c-fos, and (5) human c-fes/fps. Under stringent hybridization condition, homology was observed between hHC with c-fes > c-raf > c-fos > k-ras in decreasing degrees of relatedness.

2. The normal human liver homolog to hHC and a comparison of their restriction map. Using [^{32}P]hHC probe in Southern blot hybridization, we have identified a HindIII-HindIII DNA fragment from the normal liver DNA. After screening more than several thousand clones, one promising clone (nh1) measuring 2.95 kb was used for comparative analysis with hHC.

A restriction endonuclease map of hHC has been constructed for 12 enzymes, five of which also cut nh1 and mapped in similar relationships with each other in the termini. This observation suggests that significant alterations, e.g. recombination, might have occurred within the central portion of nh1 to account for the present hHC sequence.

3. Nucleotide sequence of hHC (PM-1 clone). We have resolved the complete nucleotide sequence of the positive strand of hHC using a combined strategy of Maxam-Gilbert method, Sanger's dideoxy sequencing technique and synthetic primer-M13 sequencing technique. The nucleotide sequence confirmed our established restriction sites along the hHC genomic map and also verified the accuracy of our sequence.

Our nucleotide sequence data showed the existence of a probable promoter region at one terminus bearing considerable homology with the published human endogenous virus ERV3 LTR sequence when analyzed by the nucleotide sequence alignment program (Nucaln of Gensrch). The hHC promoter also included a palindrome clearly marked by two long inverted repeats of TTTTTTTAAAAACCGC and AAAAAAATTTTTGGCG with the concensus enhancer sequence at the end of the loop. At approximately 60 bases from the base of the palindrome the two concensus sequences for transcription initiation are located: (1) RNA polymerase III and (2) human $tRNA_{glu}$, an identified tRNA utilized within the promoter region for VAI gene initiation. Downstream from this are the TATA box and a ATG initiation codon for an open reading frame coding for approximately 155 amino acids accounting for a protein of about 18,600 dalton.

By sequence alignment and subgenomic sequence homology search (Pustell Program), hHC was identified as a DNA sequence exhibiting homology within the human Alu gene family of liver DNA. Almost perfect alignments between two subgenomic sequences of hepatitis B virus and hHC sequence were also found, one of which is a subgenomic sequence of the proviral hepatitis B viral antigen gene, reported for PLC. Approximately 47% of the nucleotide sequence of the Tsuchida K-ras were aligned at interrupted locations with the hHC sequence, which probably accounts for the homology seen in Southern blot hybridization.

4. Possible function(s) encoded by the hHC DNA.

(a) Messenger RNA identified by Northern blot analysis and protein products obtained in cell-free translation of hybrid-selected mRNA: Our preliminary results on hHC expression study indicated the existence of a 1.8 kb and a 2.4 kb mRNA prepared from a hHC tranfected, transformed NIH/3T3 cell clone 81E: now carried as a tumor) and a 2.4 kb mRNA from MAH, hybridized to the [^{32}P]hHC probe in Northern blot analysis. This 1.8 kb or 2.4 kb mRNA, if

all functional, should be capable of coding for 600 or 800 amino acids, or a total of 72-96,000 dalton polypeptide. However cell-free translation of the hHC-hybrid-selected mRNA prepared from 81E cells revealed a unique 18,000 dalton protein and two other proteins of 40,000 and 60,000, which were also seen in total poly A-RNA directed cell-free translation. The nature and the function of these products (mRNA and proteins) awaits further work.

(b) Identification of the transforming sequence by deletion mutant analysis and by site targeted mutagenesis: We have attempted to identify the critical portion of the hHC DNA for cell transformation by subcloning hHC DNA into two unequal portions and analyzing the ability of each such subgenomic fragment in transformation of NIH/3T3 cells, i.e. deletion mutant analysis. Results obtained in this approach thus far indicated that neither the 5' Hind III-PstI 1.0 kb subgenomic fragment nor the 3' Pst I-Hind III 2.1 kb subgenomic fragment alone succeeded in transforming NIH/3T3 cells. That the 1.0 kb fragment is not responsible for cell transformation but rather function in the manner of a promoter, was strongly implied by the observation that replacement of this fragment with a murine Moloney sarcoma virus LTR sequence resulted in successful transformation of NIH/3T3 cells. The critical sequence responsible for cell transformation is thus most likely associated with the 2.0 kb fragment. As a parallel approach site-targeted mutagenesis was carried out by inserting a 10 bp BamHI linker at the PvuII site, which should create a frame-shift downstream towards the 3' terminus, and likewise, a 20 bp polynucleotide sequence reading EcoRI.dG.BamHI.dG.EcoRI, specifically synthesized and ligated at the EcoRI site within the 2.0 kb fragment and thus creating a frame-shift from EcoRI downstream. These site-targeted mutagenized clones, identifiable by the newly created BamHI site, are currently used in transfection analysis on NIH/3T3 cells. Although further studies must be carried out to warrant the conclusion, results thus far suggested that interruption of the reading frame within the 1.2 kb at the 3' terminus resulted in the loss of cell-transformation capability.

Since our DNA transfection study was always scoring at poor efficiency (5-20 ffu/5-10 ug DNA versus 10-50 ffu/120 ng of H1 DNA), we are attempting to isolate other high efficiency hHC clones from DNA prepared from secondary and tertiary transformed cell clones or tumors carried from the original study.

5. Preferential binding of AFB_1-epoxide with dG residues along the PM-1 DNA and activation of the oncogene. AFB_1, when consumed and metabolized by liver mixed function oxidases, became an epoxide. AFB_1-epoxide binds stoichiometrically with the deoxyguanine residue of any DNA tested until most or all dG residues become bound. However at low or limiting concentrations, i.e. femtomole/ug DNA, which corresponds to concentrations observed in vivo, and considering the secondary and tertiary structure of the native DNA, AFB_1-epoxide binds in a specific manner as in the following fashions: 1) most preferred dG when it is flanked by dC and dG; 2) moderately preferred when dG is preceded by dA or dG and followed by dC; and 3) the least preferred if dG is flanked by dA or dT and dT or dA. We have localized some of the dG targets within the PM-1 DNA preferentially bound by AFB_1-epoxide under limiting concentration of AFB_1 by sequencing AFB_1-epoxide bound DNA in a modified Maxam/Gilbert method. Computer analysis predicted only a few most preferred dG binding sites when these empirical rules are considered. On the other hand,

correlation between the significance of oncogene activation with any of the preferred dG target of AFB_1-epoxide has not yet been ascertained.

6. Purification and elucidation of the respective function of IL-2 and CCDF as biological modifiers in cellular immune defense mechanism. In a collaboration with Dr. George C. Ting of the Immunology Branch, we have purified the murine Il-2 and CCDF (a cytotoxic cell differentiation factor) by sequential ammonium sulfate precipitation, sephadex G200 gel filtration, DEAE cellulose column chromatography for Il-2 but sodium silicate powder absorption for CCDF followed by two successive HPLC (C10 and Diphenyl) column chromatography using gradient elution with acetonitrile. IL-2 was purified approximately 75 fold and CCDF was purified at least 2000 fold. The purified protein bands that constituted full activity of CCDF resided in two molecular species of 23 K dalton and 45 K daltons when assayed in a reconstituted condition. This suggests that there may exist a family of CCDF molecules as observed in murine IL-2.

Cytotoxic cells could be induced against various tumor cell targets by culturing normal spleen cells with syngeneic peritoneal macrophages and indomethacin. Antigenic or mitogenic stimulation is not essential in this induction of what we currently term lymphokine-induced cytotoxic cells (LICC), which may serve as an important cellular immune defense mechanism against spontaneous neoplasm. The effectors of LICC were found to be Thy^+, Lyt_2^- and AGM_1^-, a unique class of cytotoxic cells that is neither classical CTL nor NK cells. The precursors were, however, AGM_1^+ and Lyt_2^-, which were consistent of being NK-like cells. During the LICC induction process the sequential presence of IL-2 and CCDF at an appropiate time is required for successful activation and differentiation of the NK-like precursors into LICC.

Publications:

Yang, S. S., Taub, J. V., Modali, R., Vieira, W., Yasei, P. and Yang, G.C.: Dose-dependency of aflatoxin B-1 binding on human high molecular weight DNA in the activation of a proto-oncogene. Environmental Health Perspectives, 62: 231-238, 1985.

Modali, R. and Yang, S. S.: Specificity of aflatoxin B-1 binding to human proto-oncogene nucleotide sequence. In: Symposium on Monitoring of Occupational Genotoxicants, ed. by H. Norppa and M. Sorsa. A. R. Liss, Inc., N.Y., N.Y., pp. 147-158, 1986.

Yang, S. S., Malek, T. R., Hargrove, M. E. and Ting. C. C.: Lymphokine-induced cytotoxicity: Requirement of two lymphokines for the induction of optimal cytotoxic responses. J. Immunology 134: 3912-3919, 1985.

Ting, C. C., Yang, S. S. and Hargrove, M. E.: Lymphokine-induced cytotoxicity: Characterizations of effectors, precursors and regulatory auxillary cells. Cancer Research 46: 513-518, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04833-17 LCO

PERIOD COVERED: October 1, 1985 through September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Biological studies of various normal, virus-infected, and malignant cells

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

PI:	N. A. Wivel	Senior Investigator	LCO NCI
OTHER:	C. L. Robinson	Biolab Technician	LCO NCI

COOPERATING UNITS *(if any)*: P. M. Pitha, Associate Professor, Departments of Microbiology and Oncology, Johns Hopkins University School of Medicine, Baltimore, MD

LAB/BRANCH: Laboratory of Cellular Oncology

SECTION:

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	1.00	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.)*

It is the primary purpose of this project to study some of the pertinent factors which influence cell differentiation and malignant transformation, using techniques and approaches which range from the microscopic to the molecular level. Particular emphasis is given to those systems in which murine RNA tumor viruses or chemical carcinogens may be the transforming agent. A variety of mouse model systems are used, including methylcholanthrene-induced sarcomas, plasma cell tumors, mammary tumors, and neuroblastomas. Current projects include: 1) effects of interferon on methylcholanthrene-induced sarcomas of the BALB/c mouse with the aim of defining antitumor activity and relationship to immune response; and 2) effects of interferon treatment on NIH 3T3 cells transfected with various (Ha) ras related oncogenes.

Our results suggest that the major effects of interferon on chemically-induced sarcomas do not appear to be mediated through anticellular activity, but are related to the immune response in the host animal. A number of experiments confirm the necessity of functional T cells in order for interferon to exert its antitumor effect. Since interferon increases the expression of H-2 antigens on these tumor cells, this may serve to enhance their recognition by host T cells.

NIH 3T3 cells were transfected with either cellular or viral Ha-ras genes prior to interferon treatment. In most of the cell lines examined, interferon inhibited cell growth, reduced cloning efficiency and delayed, but did not prevent, tumors in nude mice. When in vitro oncogene expression was measured at the mRNA level, c-Ha-ras mRNA was reduced by 2 to 4 fold following interferon treatment, while the mRNA for H-2 antigen was increased by 3-4 fold.

PHS 6040 (Rev. 1/84)

GPO 904-917

Major Findings:

1. Effects of interferon on methylcholanthrene-induced sarcomas of BALB/c mice.

The observed effects of interferon on cells are the result of a sequence of events such as interaction with a membrane receptor, establishment of an antiviral state, inhibition of cell growth or modulation of differentiated functions. Interferon induced effects can be divided into four different categories: 1) membrane alterations; 2) changes in the cytoskeleton; 3) induction of new enzymatic systems; and 4) inhibition of inducible proteins. While the induction of new enzymatic proteins seems to play a major role in the antiviral effect of interferon, the relevance to the antitumor effect of interferon is not clear.

The rationale for pursuing these studies was predicated on a number of distinct but related factors. First, the clinical literature regarding the interferon treatment of various human sarcomas is less than definitive. Thus one could expect to derive a more interpretable set of data regarding properties of interferon in a controlled animal model system maintained in syngeneic mice. The methycholanthrene-induced sarcoma, Meth A meets the aformentioned requirements; it can be passed both in vivo and in vitro and passage in vitro does not attenuate the tumorigenicity of these cells. There are no known murine retroviruses or other infectious viruses associated with this tumor and thus one can easily dissect the anticellular effect of interferon from the antiviral one. By comparing the response in cell culture with that in the intact mouse it is possible to analyze the direct action of interferon on Meth A cells separately from the host mediated effects. It is of importance that there are stable membrane markers such as the tumor specific transplantation antigen (TSTA) and the H-2 antigens which are potentially susceptible to the action of interferon.

Our initial series of experiments established that in vitro treatment with a mixture of alpha and beta interferon caused cytostasis but was not cytotoxic, while gamma interferon was more efficient than the alpha-beta mixture. Cell growth was fully restored following removal of interferon from the system. When treated cells were used to challenge mice, the development of tumors could be prevented as long as the challenge dose was 10^5 cells or less. The interferon effect could be abrogated by using a dose of 10^6 cells. Meth A cells treated with alpha-beta interferon were rejected more efficiently than those treated with gamma interferon. These results suggest that the major effects of interferon on Meth A cells may not be mediated through anticellular activity, and thus a number of experiments were done to define the role of the immune response in this system.

Challenge of nude mice with virus-infected tumor cells serves as a stimulus for natural killer (NK) cell activity and leads to tumor rejection. We elected to compare the tumorigenicity of interferon treated and untreated Meth A cells in nude mice with that seen in syngeneic BALB/c mice to determine whether interferon treatment, in the absence of virus, could serve as a

stimulus for NK cell activity. After a challenge dose of 10^5 cells, both control and experimental groups had a tumor incidence of 100%. With a challenge dose of 10^4 cells, 100% of the controls and 85% of the mice receiving interferon-treated cells developed tumors. As opposed to BALB/c mice, interferon offered no protective effect in nude mice, indicating that the rejection of interferon treated Meth A cells is thymus dependent, and do not mediated by the NK system.

The dependence of the protective effect of interferon on the presence of functional T cells was further tested in mice in two ways. First, mice were immunosuppressed with cyclophosphamide at a dose just below the lethal level, but sufficient to produce long-term suppression affecting all classes of anti-body-producing cells. Under these conditions the results paralleled those seen in nude mice, i.e., the interferon effect was abolished. Second, mice were immunosuppressed with cyclosporin A, an agent which has been shown to preferentially affect T cells. Again the effect of interferon was negated.

Since many tumors in mice carry retroviruses or express retroviral antigens, we attempted to answer the question of whether infection with ecotropic virus (Moloney MuLV) could enhance or otherwise alter tumor cell recognition and whether it could amplify the interferon effect. Meth A cells were infected in vitro and after several passages it was established that 95-100% of the cells were producing virus. When transplanted into syngeneic mice such infected cells were rejected even without prior interferon treatment. This host response to infected cells was probably related to the antigens associated with Moloney MuLV. Friend, Moloney, Rauscher and Rich viruses are all antigenic in the mouse and vaccination has resulted in protection against challenge with active virus. Conversely, Gross MuLV does not elicit such an antibody response. Accordingly, Meth A cells were infected with a B-tropic variant of Gross MuLV, 1802-B, which is infectious in BALB/c mouse cells. When Meth-A cells infected with this virus were used to challenge mice, the results were the same as with uninfected controls.

Our recent results indicate that interferon treatment of Meth A cells increases the expression of H-2 antigens. Since the products of the major histocompatibility complex have a critical role in the initiation and regulation of the immune response, it is possible that at least some of the effects of interferon on these particular tumor cells may be due to alterations in the H-2 complex. Results from other laboratories suggest that H-2 antigens play a role in the host recognition of the TSTA of methylcholanthrene-induced sarcomas. It has to be considered that increased H-2 expression could facilitate the recognition of Meth A cells by an appropiate subpopulation of T cells.

Current efforts involve the use of an in vitro cytoxicity assay to aid in the identification of splenic T cells which have the capacity to lyse Meth A cells. Initial efforts failed to demonstrate effector T cells, but present experiments include the pretreatment of Meth A cells with such agents as concanavalin A and neuraminidase in order to make them more susceptible to lysis. There is some preliminary evidence that peritoneal exudate cells, when properly stimulated may be more effective than spleen cells in attacking methylcholanthrene-induced sarcomas, and these findings are being repeated in

our laboratory.

2. Effect of interferon treatment on NIH 3T3 cells transfected with ras oncogenes. Under certain conditions interferon can be shown to cause a reversion of NIH 3T3 cells which have been transformed by oncogenes, and there are data which suggest that interferon can prevent stable integration and expression of transfected plasmids containing thymidine kinase or dihydrofolate reductase genes. In order to further characterize these putative properties of interferon, we have analyzed several lines of NIH 3T3 cells which have been transfected with ras-related oncogenes.

The cell lines which were examined had been transformed with the v-Ha-ras gene or with the mutated proto-Ha-ras gene from the human bladder carcinoma cell line, T-24; the latter gene was under the control of an SV40 promotor. In essentially all of the cell lines examined, in vitro interferon treatment inhibited cell growth, reduced the cloning efficiency and decreased colony formation in soft agar. When treated cells were transplanted into nude mice, there was a delay in the appearance of tumors as compared to the controls, but there was no reduction in incidence.

Alteration of oncogene expression was determined by hybridizing ^{32}p-labeled DNA probes of several oncogenes to the RNA of the various transformed NIH 3T3 cell lines. The following oncogenes were tested: C-Ha-ras, v-Ha-ras, c-myc, c-myb, v-fos, and p^{53}. RNA was assayed following 48 hours of treatment with alpha-beta interferon and again after two weeks of treatment. The levels of c-Ha-ras and c-myc RNA were reduced 2 to 4 fold following long term treatment with interferon while H-2 RNA levels increased. There was no effect on any of the other oncogenes.

Although our experiments did not detect any reversion of previously transformed cells, the timing of interferon treatment was significant. If NIH 3T3 cells were pretreated with interferon prior to transfection with v-Ha-ras DNA and then treated post transfection for 92 hours, there was a 6 fold reduction in the number of transformed foci. If interferon treatment was continued for 2 weeks, transformed foci were reduced 15 fold. This reduction could not be explained by simple inhibition of cell growth, and thus interferon may have a role in inhibiting the phenotypic expression of transfected oncogenes. Experiments in progress are directed toward defining some of the mechanisms that account for these results.

Publications:

Wivel, N. A., Glasgow, G., Vengris, V. E., and Pitha, P. M.: Relationship of T-cells and retroviral infection to the antiproliferative properties of mouse interferons. Int. J. of Cancer 36: 137-142, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09051-01 LCO

PERIOD COVERED: October 1, 1985 through September 30, 1986

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*: Biology of fps/fes oncogenes

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

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 at elucidating the biological function(s) of the normal human c-fps/fes proto-oncogene, and to understand the molecular and cellular basis of its oncogenic potential. We have demonstrated that a cloned human genomic DNA fragment containing c-fps/fes-homologous sequences contains the entire coding sequence of the human c-fps/fes-encoded protein. Our results confirmed that this protein is the 92 Kda cellular tyrosine kinase that we have previously identified as the gene product of human c-fps/fes. Using a retroviral shuttle vector system, we have been able to recover an intron-less copy of human c-fps/fes DNA.

We have also identified and begun characterization of a new cellular tyrosine kinase present in all living cells that is immunologically related to the c-fps/fes product, but is encoded by a different gene.

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

Major Findings:

1. Human c-fps/fes proto-oncogene. Using fps/fes-specific antisera, we have previously identified the gene product of human c-fps/fes as a 92,000 dalton protein (NCP92) that is associated with a tyrosine-specific protein kinase activity. The expression of NCP92 was found to be tightly regulated and restricted to normal and leukemic myeloid cells. The patterns of expression of NCP92 in certain leukemic cell lines suggest that NCP92 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)]. As part of our effort to define the function of NCP92 in normal and leukemic myeloid cells, we have extended our studies to the gene encoding NCP92. Human genomic DNA sequences homologous to viral fps/fes have been molecularly cloned in several laboratories. This cloned c-fps/fes DNA consists of a 13 kb EcoRI genomic DNA fragment. We first investigated if this DNA contained the entire coding sequence of NCP92. The 13 kb DNA fragment was inserted into a eukaryotic expression vector containing the neomycin resistance gene. This plasmid was transfected into NIH 3T3 cells, tranfectants were selected in G418, and then assayed for the presence of NCP92 using specific antisera. The transfectants expressed up to 100 times higher levels of NCP92 and its associated protein kinase activity, compared to NIH 3T3 cells that had been transfected with a control plasmid lacking c-fps/fes sequences. This directly demonstrated that NCP92 is indeed the gene product of human c-fps/fes, and that the genomic DNA we used contained the entire coding sequence of NCP92. The initiation site of NCP92 is most likely an ATG codon located 1 kb downstream from the 5' end of the 13 kb fragment. This site is also where the homology between human and chicken c-fps/fes starts.

Our second undertaking was the isolation of an intron-less c-fps/fes gene derived from genomic c-fps/fes. For this purpose we removed the poly (A) addition signal from the 13 kb DNA fragment, and we inserted the resulting DNA into a retroviral shuttle vector containing the neomycin resistance gene. This plasmid was transfected into the packaging deficient cell line ψ-2, and neomycin resistant colonies expressing high levels of NCP92 were selected. From these cells, a plasmid containing an intron-less c-fps/fes fragment of the expected size and structure was isolated via cos cell fusion. This intron-less c-fps/fes DNA will be an invaluable reagent to study the biological functions of NCP92 in normal and leukemic myeloid cells, and to determine if the human c-fps/fes gene can be activated to become oncogenic.

2. Identification and characterization of a c-fps/fes-related cellular tyrosine kinase. To identify new oncogenes of the src gene family, we have raised antibodies directed against an amino acid sequence in the conserved kinase domain of the v-fps protein. The antiserum we obtained recognized avian and mammalian fps/fes proteins and, in addition, it crossreacted with a new cellular protein of 94,000 daltons (NCP94). NCP94 was associated with a protein kinase activity specific for tyrosine residues. Tryptic fingerprint analysis showed that NCP94 and NCP92$^{c\text{-}fps/fes}$ are not structurally related and that therefore, NCP94 is encoded by a gene different than c-fps/fes. NCP94 was found to be expressed in all tissues and cell types examined, and in particular, in rapidly developing embryonic tissues. Among the different tissues, the levels of NCP94 were highest in brain, stomach, and spleen, in

both the embryo and the adult animal. NCP94 is a new cellular tyrosine kinase of the src gene family that is likely to be involved in the function of all cells. Although the gene encoding NCP94 has apparently not been transduced by retroviruses, NCP94 may have oncogenic potential. This question will be addressed after cloning of the gene encoding NCP94.

Publications:

Feldman, R. A., Gabrilove, J. L., Tam, J. P., Moore, M. A. S., and Hanafusa, H.: Specific expression of the human cellular fps/fes-encoded protein NCP92 in normal and leukemic myeloid cells. Proc. Natl. Acad. Sci. USA 82: 2379-2383, 1985.

Feldman, R. A., Tam, J. P., and Hanafusa, H.: Antipeptide antiserum identifies a widely distributed cellular tyrosine kinase related to but distinct from the c-fps/fes-encoded protein. Molecular and Cellular Biology 6: 1065-1073, 1986.

SUMMARY REPORT

LABORATORY OF GENETICS, DCBD, NCI

October 1, 1985 through September 30, 1986

The laboratory is organized into a cooperative group of investigators, each encouraged to develop his or her own independent area of research centered about the common theme of how specific genes and their products determine and regulate biological functions that are involved in neoplastic development. A segment of the work is devoted to a continued understanding of immunoglobulin genes. The laboratory is supported by a contract facility maintained at Hazleton Laboratories (NO1-CB-25584), which raises many rare strains of inbred, congenic and wild mice as well as providing a source of tumor induction studies and tumor tissue used in the Laboratory of Genetics.

The laboratory meets weekly from September to May to discuss and review work in progress and on a separate day with John Minna's group to conduct a seminar on oncogenes and other basic genetic problems relevant to neoplastic development. The laboratory actively trains young investigators and graduate students in molecular and mouse genetics. The laboratory sponsored two very exciting workshops on "The Wild Mouse in Immunology" on November 4-6, 1985, and "Mechanisms in B-Cell Neoplasia IV" on March 24-26, 1986. Both workshops formulated new directions and established valuable collaborations.

A. Plasmacytomagenesis (Drs. Potter, Mushinski, Rudikoff, Nordan, and Ms. Brust)

A major project in the Laboratory of Genetics to which many separate studies address is to determine the basic pathogenetic mechanisms that are involved in the formation of plasma cell tumors in mineral oil treated BALB/c mice. The model system is a relevant experimental model for many forms of leukemias and lymphomas in man, as virtually 100% of the tumors are associated with a non-random chromosomal translocation. In addition, susceptibility to plasmacytoma induction in mice is genetically determined and is not a general characteristic of the species.

Progress this year has been made in the following areas.

1. In collabaoration with Dr. K. Sanford we have found that plasmacytoma susceptible BALB/cAn mice slowly repair double-stranded chromosomal breaks when compared with plasmacytoma resistant DBA/2 or CDF_1 hybrids. This may be the first evidence of DNA-repair defect in mice. We are pursuing this to try and determine the underlying genetic basis for the difference.

2. We have found that plasmacytoma development in BALB/c is preceded by the appearance of foci of proliferating plasma cells that arise throughout the latent period, while comparable lesions do not or rarely develop in resistant strains. Thus, susceptibility is correlated with pre-neoplastic changes and indicates susceptibility and resistance genes are exressed very early in plasmacytoma development.

3. Drs. Potter and Mushinski have found (in collaboration with Drs. H. C. Morse, III, and U. Rapp) a new method for accelerating plasmacytoma induction in BALB/cAn mice by infecting mice 7 days after a single injection of pristane with transforming retroviruses containing specific types of modified myc oncogenes. Thus far, the retroviral construct J-3 (prepared by Dr. Rapp) has been identified that induces the expected yield of plasmacytomas with short latent periods. J-3 carries a hybrid avian v-myc gene derived from the MH2/MC29 viruses. In contrast, other viral constructs carrying myc sequences induce predominantly myeloid tumors. We are carrying out experiments to find the basis for the lymphocytic tropism. Most pristane/J-3 induced plasmacytomas lack chromosome 15 translocations but produce v-myc transcripts. The method should allow us to reconstruct critical biochemical changes in plasmacytomagenesis by supplying appropriate genes.

4. Drs. Nordan and Rudikoff have purified a factor required for plasmacytoma growth in vitro to apparent homogeneity. Experiments are currently in progress to obtain amino terminal sequence data which will be used to generate a synthetic oligonucleotide probe for gene cloning. Attempts are also underway to raise monoclonal antibodies to this factor.

B. Role of cellular oncogenes in neoplastic development (Drs. Mushinski, Shen-Ong, Wolff, Mr. Bauer and Mr. Vacchio)

The Laboratory of Genetics is involved in a number of projects that are aimed at characterizing oncogene deregulation in neoplastic cells with the chief emphasis on c-myc, c-myb and ras genes.

1. Dr. Shen-Ong has further characterized a series of myeloid tumors (formerly called ABPL) which have Moloney leukemia virus inserts in the 5' end of the c-myb locus. She has demonstrated these are myelomonocytic cells and, further, that when stimulated by factors that normally induce terminal differentiation, the abnormal myb genes are activated, thus apparently keeping the cells in a proliferative mode. Active myb transcription is thought to maintain myelomonocytic cells in a proliferative mode.

2. Dr. Mushinski and Mr. Bauer have studied two unusual plasmacytomas that produce excessive amounts of very large c-myc transcripts. They have determined the molecular basis of this phenomenon by demonstrating the c-myc genes in each of the tumors carry a mutation, one in the form of a deletion, the other in the form of a point mutation that disrupts the splice donor site in the c-myc gene. These mutations exert their effects during post-transcriptional stages of c-myc expression by prolonging the half-life of the myc RNA.

3. Dr. Mushinski and Mr. Vacchio have developed a bacterial expression vector for a mouse c-myc gene and have produced a mouse myc protein that has been used to successfully immunize rabbits. This will greatly facilitate our efforts to study the product of the c-myc gene in mouse plasmacytomas and to correlate the amount of myc product with the genetic dysregulation.

4. Dr. Wolff has incorporated a c-myc derived from normal mouse spleen into a retrovirus vector and infected-pristane-primed BALB/c mice. Mice injected with this virus develop neoplasms of the myelomonocytic lineage.

C. Immunoglobulin genes and antibody diversity (Dr. Rudikoff and Mr. D'Hoostelaere)

1. Genes encoding members of a kappa chain subgroup have been cloned from recombinant DNA libraries generated in six different wild mouse species. Sequence analysis of these genes is currently in progress. Results from these studies have permitted an assessment of multigene evolution during modern speciation and provided insights into mutational mechanisms that operate oncogenes in families. (Dr. Rudikoff)

2. Light chains from antibodies to β(1,6)-galactan are unusual in that they express an Ile at the point of V_K-J_K recombination. This amino acid cannot be generated by sequences usually found at the 3' end of V_K genes or 5' to J_K genes. To determine whether nucleotides generating this amino acid were germ encoded or added during recombination, the germline gene and cDNA encoding this variable region were cloned and sequenced. Results of these studies confirm that the codon for the unusual Ile is in the germline immediately 3' to the normal termination of the V_K coding sequence. (Dr. Rudikoff)

3. Mr. D'Hoostelaere has established a partial order of the genes in the mouse kappa light chain locus through the identification of recombinants found in the laboratory and in wild mice.

D. Characterization of antibody-protein interactions (Dr. Smith-Gill and Mr. Lavoie)

Much of our knowledge of how antibodies interact with antigens has been based on antibody:hapten interactions. A large number of antibody molecules bind to proteins. The problem of characterizing the nature of these interactions is made possible by preparing monoclonal antibodies to proteins of defined structure and identifying the epitopic regions on the antigenic protein and then studying the interaction either directly (by crystallization of complexes) or indirectly (by model building).

1. Dr. Smith-Gill and Mr. Lavoie have now determined the primary structures of three monoclonal antibodies to hen egg white lysozyme, and using data from comparative binding of different avian lysozymes and chain recombination studies done is collaboraton with Keith Dorrington, the respective epitopes on HEL have been identified.

2. In collaborative studies with Dr. D. Davies, crystallization of the Fab or Fab-HEL complexes have produced suitable crystal structures of HyHEL-5-HEL that can be used to directly define the interaction.

3. Excellent progress, however, has been made in the indirect approach. Using the primary structures of HyHEL-10, a physical model of the Fv region has been constructed and refined by computer programs. The HyHEL-10 has been oriented to the HEL using the critical amino acids, and highly complementary fitting has been found. This has been energy minimized by a computer program designed by Dr. B. Brooks, and analysis of the interaction has revealed

extensive complementary topographical regions on the surfaces of both monoclonal antibody and antigen. These have involved in some regions amino acid side chain-backbone interactions. This indicates the extraordinary specificity and strength of monoclonal antibody-protein antigen interaction.

E. Mechanisms by which retroviruses (MuLV) induce erythroid transformation (Drs. Ruscetti and Wolff)

Investigations on the acute erythroleukemia-inducing virus, spleen focus-forming virus (SFFV), have concentrated on determining which viral sequences are responsible for pathogenicity and erythroid cell specificity.

1. Drs. Ruscetti and Wolff have shown that sequences in the long terminal repeat (LTR) region of SFFV do not determine the erythroid cell tropism of the virus and that expression of the SFFV env gene in the absence of other SFFV genes and the SFFV LTR is pathogenic. Thus, the primary effect of SFFV on erythroid cells, which is to alter the requirement that the cells have for the erythroid hormone erythropoietin, is due to the product of its env gene.

2. To further understand how the viral env gene product may do this, Drs. Ruscetti and Wolff have been studying $SFFV_P$ and $SFFV_A$, which both induce acute erythroleukemia in mice but differ in their effects on erythroid cells as well as in the post-translational modification of their env gene products. By making recombinants between $SFFV_P$ and $SFFV_A$, they have been able to localize the sequences responsible for the biological and biochemical differences between these two viruses to a 678 bp region in the 3' region of the envelope gene.

3. Studies on the genetics of susceptibility to early erythroleukemia induced by Friend murine leukemia virus are concentrating on a gene previously identified on chromosome 5 that encodes a viral envelope protein related to that of mink cell focus-inducing virus. Attempts are being made to confer resistance to leukemia by genetic transfer of this gene as well as to apply this mouse model for resistance to the treatment of human diseases such as AIDS.

F. Study of lymphoproliferation (Drs. Davidson and Rudikoff)

Dr. Davidson has recently joined our laboratory where she is continuing her work on the study of genes that induce lymphoproliferation and in some settings trigger autoimmunity. She has shown that the lpr and gld genes in the mouse exert their primary action in T-cells and is attempting to identify the critical genes and their mechanisms of action.

G. Culture of Olfactory Neurons (Dr. Coon)

It is the purpose of this project to analyze and develop new and difficult systems for cell culture. Dr. Coon is now pursuing intensively a single cell system: culture of cells from the neonatal rat olfactory epithelium (OLFE). The projects involving thyroid gland reconstruction and thyroid cell genetics have been postponed. He hopes to continue these timely experiments as soon as the principal collaborator, Dr. F. S. Ambesi, can return to the

laboratory. Meanwhile, the progress with the OLFE has been sufficiently exciting during the past year that it has merited our full attention. Using complex media and substrates Dr. Coon has 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 clones from 3rd to 6th passage cultures. He has initiated a hybridoma screen using OLFE as antigen and fluorescent anti-mouse IgG staining of frozen sections of OLFE for selection. These results, too, are very encouraging; many of the hybridoma supernates appear to identify small patches of cells selectively in the OLFE. The sensory neurons of the olfactory epithelium are renewed throughout life from a stem cell population. Development of this system would make available the first mammalian neuroblast to neuron cell culture system. It is hoped that basic issues in olfactory sensory physiology can be explored with this system.

H. DNA Damage and Repair in Murine Plasmacytomagenesis (Dr. Shacter)

Since joining the Laboratory of Genetics Dr. Shacter has isolated murine poly(ADP-ribosyl)transferase (pADPRT) to near homogeneity and is currently preparing monoclonal and heterologous antibodies to this enzyme. pADPRT is potentially linked to DNA repair processes. Dr. Shacter hopes to clone this gene and to be able to quantitatively study its activity in different strains of mice that vary in their susceptibility and resistance to plasmacytomagenesis. Dr. Shacter is also studying the production of oxygen radicals in pristane-injected mice to determine quantitatively the effects of pristane phagocytosis on radical formation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05596-17 LGN

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pathogenesis of plasma cell neoplasia: characterization of antigen-binding proteins

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

P.I.: M. Potter	Chief, Laboratory of Genetics	LGN,NCI
E.B. Mushinski	Bio. Lab Technician	LGN,NCI
L. D'Hoostelaere	Biologist	LGN,NCI
E. Shacter, K. Huppi	Staff Fellows	LGN,NCI
K. Sanford	Chief, In Vitro Carcin. Sect.	LCMB,NCI
S. Brust, R. Duncan	Biologists	LGN,NCI
J.F. Mushinski	Senior Investigator	LGN,NCI

COOPERATING UNITS *(if any)* Dr. H.C. Morse, III, NIAID; Dr. F. Wiener, Karolinska Insti., Stockholm, Sweden; Dr. A.O. Anderson, Mucosal Immunity Lab., USAMRIID; Dr. J. Hilgers, Netherlands Cancer Inst., Amsterdam; Dr. R. Parshad, Howard U., Wash., DC; Dr. K. Marcu, SUNY, Stony Brook, NY; Dr. U. Rapp, FCRC, Frederick, MD

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The major project in the laboratory is to determine the pathogenetic mechanisms involved in the development of mineral oil induced plasmacytomas in BALB/c mice. BALB/c mice are highly susceptible to developing these tumors while most other strains are resistant. BALB/c.DBA/2 and BALB/cAn.BALB/cJ congenic strains are being developed as model systems. The source of 'mutagenic' (carcinogenic) substances in this system are thought to be oxygen and lipid radicals that are generated by the phagocytosis of the oil and the agents that induce chromosome breaks, the consistent chromosome 15 translocations and the c-myc gene deregulation that occurs in over 95% of plasmacytomas. In collaborative studies with Katherine Sanford we have found that BALB/cAn mice have a defective ability to repair x-ray and light induced double stranded DNA breaks. We are attempting to find the genetic basis for this phenotype. A study of the histogenesis of plasmacytoma development has been completed which has shown progressive plasma cell proliferative lesions develop in BALB/c but not plasmacytoma resistant strains: BALB/cJ, C57BL/6, DBA/2, CDF_1. A new rapid induction of plasmacytomas method has been developed which involves infecting pristane conditioned mice with recombinant retroviruses containing oncogenes.

GPO 914-918

Project Description

I. Pathogenesis of plasma cell tumors

A. Objectives

To define specific steps or events in plasmacytoma development.

B. Project Description

To better define how susceptibility (S) and resistance (R) genes operate, it is important first to define critical events in plasmacytoma development.

1. Development of plasma cell proliferative foci (a preneoplastic change)

We have just completed an extensive study of the histogenesis of plasma cell tumors in mesenteric and omental tissues in pristane treated mice that covers the period from day 25 through day 210. Proliferative foci are found as early as 25 days and develop in more and more mice during the latent period. Further, the number of foci (containing 50 or more cells) per mouse increases progressively. The morphologic evidence appears to indicate that cells in a successfully proliferating focus have the ability to spread to other sectors of the oil granuloma (O.G.) and form new foci. The phenomenon of progression, i.e., development of multiple foci in a single mouse, appears to be a characteristic of BALB/cAn mice and not C57BL/6, DBA/2, (BALB/cAn x DBA/s)F_1 or BALB/cJ.

2. Effect of chronic indomethacin treatment

Indomethacin administered in the drinking water at 20 μgm/ml during the latent period inhibits the plasmacytoma formation and the progression of foci. A first experiment has been completed where the effect of indomethacin treatment begun on different days after pristane infection was examined. Indomethacin did not appear to inhibit plasma cell migration into the OG, nor the development of 1 to 4 proliferative foci, but did appear to block the formation of multiple foci.

In collaborative studies with E. Shacter we are attempting to determine if indomethacin blocks the formation of radicals in the OG, i.e., by cells that are phagocytosing pristane droplets. In this study carried out *in vitro*, peritoneal exudate cells from pristane treated mice will be fed sonicated pristane *in vitro* and the production of oxygen radicals, H_2O_2, HClO, and superoxide anion will be assayed. PMNL will be separated from macrophages, and these cell fractions will be treated with indomethacin to determine if radical production can be inhibited.

3. Time and site of origin of chromosome (chr) 15 translocations

Since over 98% of pristane induced plasmacytomas have translocations of chr 15 and have an associated deregulation in c-*myc* gene expression, it is important to define when these translocations occur. Ms. Susan Brust, who has spent three months in Dr. Francis Wiener's lab learning how to make chromosome

spreads and karyotype cell lines, will attempt to fuse the plasma cells in OG with a drug marked Ig producing B-cell tumor (lacking a chr 15) and clone out hybrid cells that secrete IgM or IgA. These cell lines will be karyotyped to determine if the plasma cells from the OG tissue have rcpt(12;15) or rcpt(6;15). The hybrid cells should be able to secrete Ig and will be selected on their ability to secrete Ig.

4. Rapid induction of plasmacytomas in pristane treated mice with transforming retroviruses

A single dose of 0.5 ml of pristane induces plasmacytomas in 20 to 30% of BALB/c mice with minimal latent periods of 140 days and mean latent periods of 215 days. We have found that if mice are given 0.5 ml pristane and then 7 days later infected with retroviruses containing specific kinds of oncogenes that plasmacytomas can be induced in approximately the same incidence but with short latent periods ranging from 46 days post virus to a mean of 71 days post virus.

Two series of experiments are in progress. The first deals with the J-2, J-3 and J-5 viruses constructed by Ulf Rapp. The J-2 virus contains a complete but hybrid v-raf/mil component and a complete avian hybrid myc gene, its 5' half being derived from MH2 and the 3' half from MC29. The J-5 virus contains a complete MC29 avian v-myc. J-2 and J-5 induce myeloid tumors, but J-3 induces plasmacytomas. J-3 differs from J-2 only by having a 200 bp deletion in the 5' end of the v-raf/mil that disrupts the reading frame of the raf/mil gene. Further, the role of the incomplete v-raf/mil is not known. Experiments are in progress to determine the structural basis of the plasmacytomagenic specificity. An important conclusion, however, is that the target cell for plasma cell transformation is generated within 7 days. A second important characteristic of J-3-pristane induced plasmacytomas is that 80% and probably more of them lack chr 15 translocations. Thus, the avian v-myc sequences appear to replace the requirement for the chr 15 translocations that deregulate c-myc. We previously described the co-plasmacytomagenic activity of Abelson virus, but the Abelson virus-pristane induced plasmacytomas had translocations and, therefore, it was assumed Abelson virus was transforming a cell that had already undergone translocation. A third feature of the J-3-pristane tumors is that their latent periods are intermediate. Since it is assumed the effect of the v-myc is immediately established, the time required for full transfomation requires a second event.

In a separate series of experiments using the same protocol, done in collaboration with Kenneth Marcu at Stony Brook, we are testing the co-plasmacytomagenic activity of another series of myc and ras gene retroviral constructs. In the first series of experiments we have found that a construct containing H-ras + mouse c-myc + Eμ (the heavy chain enhancer sequence) induces plasmacytomas with short latent periods. Further, we have found one tumor with a virus containing ras only. The experiments are in progress to confirm and extend this observation.

This mouse plasmacytoma induction model should permit us to artificially supply a second deregulation that transforms the cells to full malignancy and, hopefully, from this information we may be able to identify a second naturally developing deregulation in pristane plasmacytomagenesis.

II. Identification of genes that determine susceptibility to plasmacytomagenesis in BALB/c mice and resistance in other strains

Professional Personnel:

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

Objectives:

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

Methods Employed:

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

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

1. Identification of resistance genes in DBA/2 and phenotypic difference relating to the repair of double stranded chromosome breaks

A first approach in identifying DBA/2 R genes was to determine if PCT-R genes are linked to known markers, or relevant phenotypic characteristics that distinguish DBA/2 from BALB/c. A series of BALB/c.D2 semicongenic stocks were constructed by backcrossing D2 markers or phenotypes onto BALB/c for 6 or more backcross generations. Over 10 such stocks have proved not to contain a linked PCT-R gene. Several of these stocks, i.e., those carrying $Fv\text{-}1^{n}$ (chr 4) and Qa2 (chr 17) have shown a weak partial resistance. We have now constructed a $BALB/c{\cdot}D_2Fv\text{-}1^{nn}Qa2^{++}$ mouse to determine if the two partial resistance genes will give strong resistance. One candidate for a strong R gene effect is with a stock bred for the gene Tol-1 phenotype. Unfortunately the assay for this phenotype is erratic and unreliable and the location of the genes is not known. Fortuitously N8 progeny have been obtained and these have shown evidence of strong resistance. We are attempting to find a genotypic correlate by screening these mice against a wide variety of discriminating probes.

The major experimental problems in accomplishing this goal is identifying resistant mice by an induction assay. This process requires 250 days. However, we have a new potential method for identifying R mice by using preplasmacytoma focus assay which can greatly shorten the time required to assay mice for susceptibility or resistance. Using methyl green pyronin stained sections, a large sample of the mesenteric and omental connective tissues can be screened for foci that contain 50 or more cells. Experiments have now been completed and submitted for publication on the morphology and quantitation of foci in susceptible BALB/cAn and resistant BALB/cJ, C57BL/6, DBA/2 and (BALB/cAn x DBA/2)F_1 mice through day 200 post pristane. It has been found that in all strains plasma cells continuously migrate into the OG in moderately large numbers, but only in BALB/cAn mice is there a progressive increase in the number of foci in individual mice as well as an increase in the number of mice that develop foci. These data based on samples of 20-25 mice at each time point make it possible to determine if mice are susceptible or resistant to plasmacytoma development.

In collaboration with Katherine Sanford and Ram Parshad we are attempting to identify the genes responsible for a defective repair of X-ray induced double stranded chromatid breaks in BALB/cAn mice. When LPS blasts (B-lymphocytes) of BALB/cAn mice are irradiated in vitro, they develop double stranded chromatid breaks that are very slowly rejoined. In contrast, the plasmacytoma resistant DBA/2 and (BALB/cAn x DBA/2)F_1 repair these breaks efficiently within two hours. These results, which have now been confirmed in multiple experiments, are being prepared for publication. Our future work will be to show that BALB/c·D2 (plasmacytoma resistant) mice do not have this defect and to identify the specific gene involved. Emily Shacter, Konrad Huppi and Beverly Mock will attempt to develop a biochemical assay for chromosome breakage.

2. Identification of resistance genes in BALB/cJ

The resistance of BALB/cJ has been confirmed by the focus assay method where it has been found that BALB/cJ develop far fewer persisting foci by day 200. BALB/cJ is known to have most of the same allelomorphic genes as in other BALB/c lines including BALB/cAnPt; there are, however, a number of phenotypic differences and a few genotypic, none as yet have relevance to tumor formation. One of the phenotypes is the Raf-1^b allele (a recessive gene) that determines a high serum level of alphafetoprotein in adult life. We are constructing a BALB/cAnPt·BALB/cJ Raf-$1^{b/b}$ congenic to determine if Raf-1^b is linked to resistance. BALB/cJ has at least 5 other phenotypic differences from other BALB/c sublines, and it has been suggested by Leslie Kozak of the Jackson Lab that BALB/cJ may have a major regulatory gene mutation that has multiple pleiotropic effects. We obtained preliminary evidence that Raf-1^b is linked to a second phenotype that regulates the expression of several of the components of MUP. Dr. T.H. Roderick of the Jackson Laboratory described these differences, and we have now confirmed his observation using the more sensitive isoelectric focusing. We have found two phenotypic differences in MUP expression: a very reduced expression of band 6 (called 6^{low}) and an absence of bands 6, 7 and 8 (nul phenotype). In addition, Konrad Huppi has found a restriction fragment polymorphic difference in the MUP locus on chr 4 that distinguishes BALB/cAn from BALB/cJ. Using our congenic mouse BALB/cAn·JRaf-$1^{hi/hi}$ mouse, we have

found that these mice have the chr 4 genotype of BALB/cAn but the 6^{lo} phenotype of BALB/cJ, indicating that they differ by a gene that regulates MUP levels. Using crosses to wild mice we are now trying to identify the chromosomal location of this regulator gene and then to determine if this gene itself or one closely linked to it is responsible for resistance to plasmacytoma development. Mr. Rob Duncan, a graduate student is carrying out this analysis.

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

In collaboration with Dr. Konrad Huppi, DNA subtraction hybridizations have produced DNA probes that distinguish genomic DNA from BALB/cAnPt and BALB/cJ. The first of these, the π19 probe detects multiple restriction fragment bands. This new approach should provide a number of new markers.

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

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

Significance to Biomedical Research and the Program of the Institute:

Many forms of neoplastic development, including viral, chemical, physical, and unexplained models have a genetic influence that determines susceptibility or resistance. Very few of these systems are condusive to genetic analysis, but the induction of plasmacytomas in BALB/c mice offers an experimental system for approaching some of these questions. First, BALB/c and NZB are unique among the inbred strains by being highly susceptible for developing this rare type of tumor. Second, the inducing agent in question is not metabolically active, thereby eliminating a metabolic pathway. Pristane does initiate a chromic inflammatory process (probably associated with the production of oxygen radicals), and this is very relevant to certain forms of human tumors. For example, endemic Burkitt's lymphoma in man is associated with chromic malaria, a process known to stimulate phagocytosis of dammaged rbc in the spleen. Third, the BALB/c system is very likely not dependent on the activation of endogenous MCF and ecotropic retroviruses to its development. Fourth, the BALB/c plasmacytoma system is associated with the development of non-random chromosomal translocations is an experimental model for the growing number of human hematopoietic tumors that are associated with non-random chromosomal translocations. The identification of specific genes, and their path of action in an experimental model of tumor development should elucidate critical events in neoplastic development but also identify genotypes that are at higher risk for tumor development. This may provide critical information that could be extrapolated to certian forms of human tumor development.

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

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

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

Objectives:

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

Major Findings:

Production of the CK^a-Hd recombinant mouse resulted in a gene order centromere-Tcrb-3.4 Cm-Hd-4.5 Cm-CK. Analysis of the Igk recombinant mouse "NAK" and two B6.PL Lyt2 congenics produced the following gene order: (VK1, VK9, VK11, VK23, VK24, pC9-26)-(VK4, VK8, VK10)-(7S RNA, VKser)-(VK21, Lyt2). The orientation with respect to the centromere and outside markers was not elucidated. Recent studies on inhouse produced recombinants have placed the homeo box gene (Hox-1) very close to Hd1.

The Igk locus in inbred mice is relatively nonpolymorphic at the level of restriction endonuclease fragment (REF) length polymorphisms. There are two CK alleles and from 2 to 4 alleles for each of the VK gene groups listed above in the 60+ inbred strains tested. The wild mice held on the support contract and recent samples obtained from Dr. R. J. Berry (University College, London) have provided us with a large number of new alleles. There appears to be a 4 fold increase in the number of VK alleles in commensal and feral populations of mice. Besides the increased diversity implied by these new allelic forms, the wild mice provide direct evidence of recombination in natural population, and the interpretation of the allelic segregation patterns should imply a gene order.

Significance to Biomedical Research and the Program of the Institute:

There are separate coding regions for the two classes of light chains, kappa and lambda. Since over 97% of the Ig molecules formed in inbred strains of mice have kappa light chains, the number of variable kappa (VK) coding genes could be considered an important component of the Ig diversity in the mouse. These studies will provide additional information concerning the organization of this multigene family and could be used as a model for comparison with other multigene families. The organization of this complex locus could lead to speculations about the methods of gene duplication. Current literature indicates VH genes may be activated in order using the genes closest to the constant region first. A mapping of Igk will provide comparisons which could be used to expand models for gene rearrangement. The wild mice will be important in determining the extent of diversity provided by allelic forms of VK and help establish which are the newest or raret forms present in natural populations.

Proposed Course of Research:

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

The ABP.AK-VK^a/VK^a-wa-1/wa-1 (1st recombinant) have been mated to the S.C.B6.C3-Hd/+ mice. The F_1 progeny which expresses the Hd phenotype have been backcrossed to the 1st recombinant parental population. The progeny which express both or lack both outside phenotype markers will be recombinant mice, and DNA from these mice will be tested for recombination within the Ig-k locus using the CK and VK region probes. Since all of the REF of the AKR mouse will be present in at least one copy in all of the N_1 progeny, only the presence or absence of unique REF can be detected. This will allow a mapping of the majority of the unique inbred REF. An additional cross involving ABP/Le-wa-1/wa-1 and C_{58}-Hd will allow a mapping of the other allelic forms of VK genes. A total of 19 recombinants have been detected between Hd and wa-1 from these populations. Wild mouse samples from different geographic locations and progeny from populations established in the laboratory are being examined for VK, CK and other chromosome 6 genetic polymorphisms.

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

Objectives:

Plasmacytomas (PCTs) are induced in genetically susceptible strains of mice by agents (e.g., pristane) that provoke a chronic inflammatory response. The precise role of inflammatory reactions in the pathogenecity of murine PCTs and the genes that control susceptibility have not been defined. However, the following observations suggest a plausible model to test: (i) almost all primary PCTs contain non-random chromosomal rearrangements which result in deregulation of the c-myc oncogene; (ii) B-lymphocytes from PCT-susceptible mice repair their DNA less efficiently in response to ionizing radiation and show higher levels of spontaneous DNA breaks than cells from PCT-resistant mice; (iii) treatment of mice with the nonsteroidal anti-inflammatory drug indomethacin strongly inhibits tumor development; (iv) pancreatic cells from PCT-susceptible mice exhibit much greater sensitivity to the DNA-alkylating agent streptozotocin (which also acts on lymphoid cells) than cells from PCT-resistant mice.

The primary objective of this research project is to test the hypothesis that the genetic basis for susceptibility to murine plasmacytomagenesis lies in the differential abilities of the B-lymphocytes to repair DNA damaged by reactive oxidants in the inflammed tissue; activated macrophages and neutrophils produce active oxygen molecules which have been shown in other systems to induce DNA damage in cells. An inadequate DNA repair system may allow for tumorigenic chromosomal translocations such as those that result in activation of the c-myc oncogene.

Two biochemical approaches are being followed to test this model:

1. Investigation of the possible role of nuclear poly(ADP-ribosylation) (pADPR) in the critical repair of DNA in B-lymphocytes from PCT-sensitive (BALB/cAn) and -resistant (BALB/cJax) mice. We are examining the comparative biochemistry and genetics of the enzyme pADPR transferase (pADPRT) which catalyzes this reversible covalent modification of DNA-binding proteins. It is thought to play a primary role in regulating DNA repair such as would be required following O_2 radical-induced damage. In addition, by co-culturing B-cells and neutrophils (resting and activated) isolated from both strains of mice and then assaying DNA damage, repair, and modulation of pADPRT activity, we can determine directly the relative severity of the oxidative bursts and the differential sensitivities of the B-cells to oxidants.

2. Direct measurement of active oxygen molecules produced by peritoneal neutrophils in response to pristane and/or phorbol ester tumor promoters. Thus, we will test (i) whether pristane induces an oxidative burst in neutrophils, (ii) for possible qualitative and quantitative differences in the reactive oxidants produced by neutrophils from susceptible and resistant mice, and (iii) for an effect of indomethacin on oxidant production and stability.

Methods Employed:

Purify pADPRT from tissues of BALB/cJax and BALB/cAn mice by standard protein purification procedures (e.g., ammonium sulfate fractionation, DNA-cellulose affinity chromatography). Assay enzyme activity by measuring incorporation into proteins of ^{32}P from radiolabeled NAD^+ (the enzyme substrate). Quantify the enzymes and assay and compare relevant kinetic parameters, such as DNA-dependence and Km for NAD. Immunize rats with murine pADPRT to prepare monoclonal antibodies. Prepare hybridomas by standard methodologies.

Isolate monoclonal antibodies by adapting positive hybridomas to growth in a serum-free medium developed previously. Employ the antibodies to (i) compare the native forms of the enzymes in vivo (e.g., immunoprecipitation, Western blots), (ii) measure modulation of enzyme levels (gene expression) in response to DNA damage, and (iii) screen cDNA libraries for a probe to compare the structure and expression of the genes from both strains of mice.

Assay for superoxide anion (O_2^-) and superoxide dismutase by the cytochrome c assay. Assay for H_2O_2 and catalase by UV absorbance spectroscopy and reduction of phenol red. Assay myeloperoxidase (MPO) by 4-amino-antipyrine reduction, and production of hypochlorous acid (i.e., the MPO-H_2O_2-halide system) by chlorination of taurine. Isolate elicited peritoneal neutrophils from BALB/c Jax and An mice and assay all of the above mediators of inflammation from oxidative bursts induced in the presence and absence of indomethacin.

In collaboration with Drs. Beverly Mock and Konrad Huppi, set up DNA damage and repair assays (e.g., alkaline elution, radiolabeling of DNA strand breaks) that will be suitable for analyses on B-lymphocytes.

Major Findings:

1. Two rats immunized with partially purified pADPRT prepared from Swiss Webster mouse spleens became antibody positive; one of these was immunized by a novel protocol employing pADPRT bound to DNA cellulose as the immunogen. Two protocols for screening hybridomas have been developed - a dot immunobinding assay of highly purified pADPRT and an automated particle concentration fluorescence immunoassay employing biotinylated pADPRT and avidin-beads.

2. pADPRT was purified to near homogeneity from BALB/cJax and An testes. The enzyme was present in comparable levels in crude extracts from both strains, and the kinetic properties of the purified enzymes (e.g., DNA and histone activation, Km for NAD) were similar but not quantitatively identical. Their SDS-polyacrylamide gel electrophoresis profiles (i.e., molecular weights) appeared the same. We have yet to compare physiological activation of the enzymes by inducing DNA damage in viable B-cells.

3. Assays for the following agents of inflammation have been successfully worked out: O_2^-, H_2O_2, HOCl, SOD, MPO, and catalase. So far, addition of

indomethacin (50 μm) has had no significant effects in vitro. However, we have found found that low levels of H_2O_2 (50 μm), indomethacin (50-100 μm), and vitamin C (100 μm) kill plasmacytoma cells in tissue culture.

4. Biochemical studies on the polypeptide growth factor (PCT-GF) required for PCT growth in tissue culture have led to the following conclusions: (a) PCT-GF cannot be replaced by extracellular cAMP, in disagreement with other reports; (b) PCT-GF stimulates a small (~ 15%) but significant decrease in intracellular cAMP (studies carried out in collaboration with Dr. Joel Moss, NHLBI). Thus, PCT-GF does not act via activation of adenylate cyclase; (c) analysis of phosphoproteins and phosphoamino acids formed in response to PCT-GF revealed a general increase in protein phosphorylation but no apparent formation of P-Tyr.

5. A serum-free medium which supports healthy growth of lymphoid cell lines in culture has been fully developed and characterized. It is simple to prepare and inexpensive and reliable to use. Cell lines grown in this medium include TEPC-1165 and MOPC-460D (PCT cell lines), P388, P388D1 and SP2/0 myeloma cells. The last provides for the possibility that hybridomas derived from them may be propagated serum-free, thereby allowing for production of relatively pure monoclonal antibodies without the need for further purification or sacrificing of laboratory animals. Importantly, TEPC-1165 cells still produce antibody in the serum-free medium (manuscript in preparation).

Significance to Biomedical Research and the Program of the Institute:

Artificial induction of inflammation and plasmacytomagenesis in the peritoneal cavities of genetically susceptible strains of mice provides an excellent model system for studying the biochemical basis for susceptibility to disease and the roles of inflammatory agents in promoting B-cell neoplasia. These studies necessarily encompass a broad range of topics of central importance to cancer research, including chromosomal translocations, oncogene activation, DNA damage and repair, and the biological production, activity and tumorigenicity of oxidants.

Proposed Course of Research:

Prepare monoclonal antibodies against pADPRT. Compare the native structures of the enzymes in susceptible and resistant strains of mice. Prepare cDNA probes to study structure and expression of the genes. Assay production of active O_2 species by neutrophils in response to pristane and test for DNA damage in target B-cells (e.g., LPS-blasts) from both strains. Determine whether the genes responsible for susceptibility to plasmacytomagenesis reside in the target B-cell populations or in the inflammatory cells (macrophages and neutrophils) or elsewhere in the immune system. Test for effects of indomethacin in all of these processes.

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

Objectives:

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

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

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

Major Findings:

Utilizing the ability of homologous DNA to reassociate following denaturation, we have succeeded in cloning probes which recognize differences between subtracted DNA samples. One category of DNA probes which we have obtained map to mouse chromosome Y as a result of BALB/c male by female hybridization

subtraction. These probes (Jax43, Jax118 and tol-20) represent useful markers for mouse chromosome Y which, heretofore has been somewhat devoid of markers. In genetic analysis using these Y chromosome associated probes, an occasional phenotypic female mouse will contain hybridizable fragments. Previous literature indicates that genetic material from mouse chromosome Y is capable of crossing over to chromosome X. The results from our DNA probes substantiate that a female breeder mouse contains genetic material originally derived from the Y chromosome. Genomic cloning and sequence analysis of this DNA indicates this region of DNA is highly conserved among mouse species as a multicopy sequence. Further studies intend to examine transcription of this region with respect to tissue specificity and developmental status.

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

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

Significance to Biomedical Research and Program of the Institute:

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

Proposed Course of Research

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

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

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08727-09 LGN

PERIOD COVERED
October 1, 1985 to September 30, 1986

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
M. Potter	Chief, Lab. of Genetics	LGN,NCI
G.L.C. Shen-Ong	Visiting Fellow	LGN,NCI
K. Huppi	Staff Fellow	LGN,NCI
S.R. Bauer	Biologist	LGN,NCI
L. Miribel	Guest Worker	LGN,NCI
L. Wolff	Sr. Staff Fellow	LGN,NCI

COOPERATING UNITS *(if any)*
K. Marcu, Dept. of Biochemistry, State Univ. of NY, Stony Brook, NY; R. Watt, Smith, Kline & Beckman, Phil. PA; H.C. Morse, LVD, NIAID; M.C. Sneller, LCI, NIAID; D.M. Klinman, A&R, NIADDKD

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

t is the long range purpose of this project to study the control mechanisms im-
ortant in regulating cell growth, neoplastic transformation, and protein synthesis
n normal and malignant cells of the immune system. To this end we are studying the
tructure of the proto-oncogenes in normal and tumor tissues from mouse and man and
he expression of these oncogenes as mRNAs and proteins in mouse plasmacytomas and
ouse and human myeloid and lymphoid tumors. In addition, we are investigating what
ole Abelson and Moloney leukemia viruses as well as recombinant viral constructs
lay in the induction of mouse tumors and alteration of their cellular oncogenes.
e have discovered that Moloney leukemia viruses frequently insert themselves as
roviruses in either the 5' end or 3' end of the mouse c-myb locus in certain mye-
oid tumors. We have isolated cDNA clones of murine and human c-myb transcripts
rom myeloid and lymphoid tumors and are characterizing these clones and the genes
ncoding c-myb in both these species. We have found that recombinant viruses con-
aining mouse c-myc genes induce plasmacytomas or myeloid tumors in pristane-primed
ALB/c mice; the nature of the viral oncogene determines the tumor type. Studies
n these and other plasmacytomas indicate that expression of myc is regulated at a
umber of different metabolic sites. We have found that the very high levels of
-myc RNA in two of these tumors result from a combination of increased transcrip-
ion of the c-myc gene and a stabilization of the c-myc RNA transcripts. We are
tudying whether these tumors with high c-myc RNA levels can constantly produce
uper abundant amounts of myc protein or if myc RNAs are subjected to translational
ontrol mechanisms. We are also studying how much translational controls operate
n immunoglobulin synthesis in B lymphocytes.

Project Description

Objectives:

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

Methods Employed:

Our experimental model systems include mouse plasma cell tumors, lymphosarcomas, myeloid tumors and autoimmune tissues. The human tissues used include normal tissues, tumors, tissue cultured lines or peripheral blood cells. The DNA is extracted and studied by Southern blotting, genomic cloning, heteroduplexing and DNA sequencing. The mRNAs are studied by electrophoresis, blotting, S1 nuclease mapping, nuclear run on studies, primer extension, R looping, cDNA cloning and DNA sequencing. Both plasmids and bacteriophage vectors are used for cloning and propagation of DNA clones in bacteria and for production of hybridization probes. Immune precipitation of metabolically labeled proteins is performed using antiserums produced to myc proteins synthesized by bacteria containing recombinant plasmids which are capable of induction of expression. Riboprobes are also being used to detect mRNAs in single cells or tissue sections by in situ hybridization.

Major Findings:

A. Expression of the myc genes is being studied in tumors that have chromosomal translocations that occur upstream of the myc promoters and do not interrupt the coding region. Most of these tumors contain modestly elevated levels of c-myc RNA transcripts that are constitutively expressed owing to a separation of the c-myc promoters from down-regulating sequences that are normally about 500 bp upstream. Two tumors in this group, TEPC 1165 and TEPC 2027, contain exceptionally high levels of c-myc RNAs which are unusually large in size as well. The large size of these myc RNAs is due to the anomalous inclusion of the first intervening sequence (IVS-1). In order to learn why post-transcriptional processing failed to splice out the IVS-1, we cloned the genomic fragments containing c-myc from both tumors and studied them by restriction mapping and DNA sequencing. TEPC 1165 was found to have a 194 bp deletion that removed the junction of myc exon I and IVS-1 including the signals for the splice donor site. There was no deletion in TEPC 2027, but there was a single base transversion in the splice donor site that changed the dinucleotide GT, invariably the first two bases in every intron, to GA. The splice donor mutation or deletion inhibited removal of the first myc intron by splicing, leading to the unusually large 4.0 kb and 3.9 myc RNAs in TEPC 2027 and TEPC 1165, respectively. In TEPC 2027 and TEPC 1165 the splice acceptor site at the junction of IVS-1 and exon II is unaffected, apparently, and available for splicing if an alternate donor splice site can be found. Both tumors contain minor smaller myc RNAs that probably represent the results of such alternate splice events utilizing secondary or cryptic splice donors. TEPC 2027 has a normal sized 2.4 kb myc RNA that probably

represents an inefficient use of the mutated (GA) splice donor. TEPC 1165 has a 3.0 kb myc RNA that contains only the sequences from the 5' portion of IVS-1, so we think that a cryptic splice donor site in the intron has been used to splice out the 3' portion of IVS-1.

The unusual size of these myc RNAs is now understood, so now we studied the causes for the high levels of these RNAs. Nuclear run on studies showed that all the myc RNAs were transcribed at a rate 4-5 times that of the 18-81 lymphosarcoma, a control tumor. The stability of these RNAs was studied by inhibiting mRNA transcription with actinomycin D and then measuring the amounts of hybridizable myc RNA found in RNA preparations harvested at different times after treatment. The normal sized 2.4 kb RNA in TEPC 2027 had the short 20 min half-life expected of myc mRNA in normal tissues. The 3.0, 3.9 and 4.0 kb myc RNAs had half-lives prolonged by as much as 20 fold. This shows that the presence of sequences from IVS-1 causes a significantly increased stability of myc mRNA. Thus, both increased transcription and increased stability contribute to the unusual abundance of myc mRNA in TEPC 1165 and TEPC 2027.

B. We have described the frequent involvement of the c-myb in virus-induced myeloid tumors. We have also described high levels of myb RNA in certain autoimmune mice and human patients. We have been anxious to learn the structure of the c-myb gene in normal and neoplastic tissues. The only myb probes heretofore available have been homologous to the 2 kb of the avian viral myb gene. We know that the c-myb mRNA in man and mice is nearly 4 kb, so there is a great deal of normal c-myb not represented in these probes. More extensive probes would enable us to examine this locus more effectively for rearrangements or altered expression in human and murine tumors. Thus, we have been isolating cDNA clones from the abundant myb RNA in mouse myeloid tumors. Since no such clones have been described for human myb, we have isolated cDNAs from normal human T lymphocytes and from B cell, T cell and myeloid neoplasms. Sequence comparison between mouse and man reveal considerable homology. The 5' end of the gene and its transcripts have been hard to reach by cloning, so its mysteries are the current target of our cloning endeavors.

C. Expression of immunoglobulin heavy chain genes and isotype switching are being studied using the mouse B cell line 70Z/3, which can be induced to undergo isotype differentiation with a Peyer's patch derived T cell hybridoma, HAJ-3. Unstimulated 70Z/3 B cells express small amounts of membrane (IgM) (mIgM) and no membrane IgG (mIgG) prior to culture, whereas 70Z/3 B cells following co-culture with HAJ-3 T cells express large amounts of mIgM and mIgG2b. Such new Ig expression is associated with the induction of IgG2b specific mRNA, but not with DNA switch region rearrangement or C_H deletion. When T cell influences are removed, the 70Z/3 cells no longer express mIgG2b but continue to express mIgM. The effect of LPS is quite different in that it stimulates 70Z/3 cells to express considerable IgG2b mRNA, but does not induce expression of detectable mIgG2b; this indicates that a T cell influence is necessary for the production of translatable IgG2b mRNA. These results are consistent with the idea that the first step of B cell isotype differentiation is a reversible, T cell-induced step in which downstream gene transcription and translation is brought about in the absence of switch recombination and deletion. We are currently using this cellular system as well as related systems to further investigate the molecular mechanisms by which T cells can regulate the early events of B cell isotype differentiation.

D. Rapid induction of plasma cell and myeloid tumors by recombinant viruses containing myc genes has been achieved in adult BALB/c mice only when the mice had been previously treated with intraperitoneal pristane. Moloney LTRs were used to promote integration and expression of 4 different forms of myc: 1) all three exons of mouse c-myc cDNA; 2) exons 2 and 3 of mouse c-myc cDNA; 3) J2 virus, containing raf/mil, MH2 and MC29 v-myc; and 4) J3 virus, the same as J2 only with the raf gene inactivated by a frame shift deletion. The first virus induced virtually no tumors. The second and third induced mostly myeloid tumors. The fourth virus (J3) produced 22% plasmacytomas plus a few myeloid tumors. In nearly all tumors the inducing virus was integrated in the genome and expressed abundantly. With rare exceptions the plasmacytomas seem to have been induced in a way significantly different from those previously described from i.p. pristane, namely there was no reciprocal translocation inolving the myc chromosome, and the c-myc was repressed rather than constitutively expressed. Apparently, abundant expression of MH2/MC29 v-myc adequately substitutes for the c-myc changes commonly associated with BALB/c plasmacytomas.

Significance to Biomedical Research and the Program of the Institute:

The goals of this laboratory and our parts of the Program of the National Cancer Institute are directed toward the understanding of the genetic and molecular events that result in cancer. Increased expression of the c-myc oncogene is associated with nearly 100% of mouse plasmacytomas and human Burkitt's lymphomas. Thus, our success in learning that increased myc RNA transcription as well as RNA stabilization increases c-myc expression in super-producing plasmacytomas contributes to knowledge of how this important molecule is regulated in normal and neoplastic cells. It will be of great importance to determine whether the high levels of myc mRNA in these plasmacytomas are paralleled by similarly high levels of c-myc protein. This will require the development of antibodies capable of precipitating mouse myc protein.

The myb oncogene is similar to myc inasmuch as its protein product is generally localized to the nucleus and its expression waxes and wanes during T cell and myeloid cell stimulation or differentiation. It is a much larger and more complex gene than c-myc, and we are getting closer to understanding its structure and the nature of its transcripts. We have determined over 3000 bases of the structure of the myb cDNA for man and for mouse. We are using this structured information to investigate lymphoid and myeloid tumors in mouse and man, which have a good likelihood of having important genetic alterations at the myc locus.

In an effort to understand more about the role of T cells in isotype switching in B cells, the system employing 70Z, a clonal B cell line, and HAJ-3, a clonal T cell, offers several unique advantages. This system is being more fully exploited to characterize the state of transcription of the different regions of the Ig heavy chain genes. It makes it possible to investigate the role of T cells in reversible expression of heavy chain isotypes other than IgM.

Proposed Course of Research:

In order to make a more definitive causal connection between myc expression and neoplasia, we have taken three approaches. The first is to induce increased myc expression in normal cells by infecting mice with myc-containing retroviruses. The results indicate that viruses containing either c-myc or v-myc can induce tumors, sometimes myeloid and sometimes plasmacytoid. The second approach uses anti-mouse-myc antibodies to examine the expression of myc protein, rather than just myc mRNA, in tumors and proliferating normal cells. The protein studies had not been possible heretofore owing to the lack of suitable antibody. The third approach examines the incipient tumors in histological sections using in situ hybridization of myc and myb mRNA and antibody staining of the protein products of these genes in order to determine the kinetics of tumor cell appearance and correlate it with expression of c-myc and the characteristic chromosome translocations.

Most of these studies are still in their infancy and need to be pursued to greater depths. It will be important to analyze more tumors induced by different myc viral constructs in vivo and in vitro in order to sort out what determines whether the virus will cause myeloid or lymphoid tumors. Studies of c-myc expression in single cells in developing tumors induced by pristane alone or pristane plus Abelson virus or recombinant viruses should shed light on the kinetics of myc activation in this system of tumor development. Myc expression seems to be under a very tight control at the level of RNA transcription and RNA breakdown. There are indications that post-transcriptional modification of RNA transcripts play important roles in stabilizing mRNAs, so these will be examined in more detail in plasmacytomas TEPC 1165 and 2027 which have structural mutations that affect splicing. Translational and post-translational regulation of myc protein abundance are other sites where the active myc protein molecules can be controlled. Our recent progress in precipitating mouse myc protein with polyclonal anti-myc antibodies will permit a more detailed dissection of this metabolism. Other proteins besides myc can be regulated at the transcriptional and post-transcriptional level as shown by our studies with Ig switching in 70Z B cells. Pursuing this aspect of protein synthesis and stabilization in plasmacytomas and 70Z simultaneously should enable us to recognize generalizations that can be made about translational control of expressin of myc and Ig genes at this level in normal and neoplastic B cells. In addition, the use of clonal T cells to control expression of RNAs and proteins in a cloned B cell affords a unique opportunity to study Ig switching at the molecular level.

As our knowledge increases about the structure of the c-myb gene, we will be better able to understand the control of its expression. We need to find clones of the 5' end of the gene to get at the control regions in it. Then we can look into neoplastic diseases of mice (e.g., the myeloid tumors discussed above) and men (such as lymphomas/leukemias with lesions in chromosome 6 q22-24, where myb has been localized) for involvement of the c-myb oncogene.

The long latent period before the plasma cell and myeloid tumors arise as full-blown malignancies suggests that the c-myc and c-myb expression alterations discussed above may not be sufficient for complete neoplastic transformation. Thus, we will embark on a search for a second event which may complement these

alterations. We will begin with a careful examination of the ras family of oncogenes by hybridizing DNA from tumors with synthetic oligonucleotides representing normal ras sequences at codons 12/13 and 61/62, known to be frequently mutated in other tumors, with dramatic consequences such as neoplastic transformation. Mismatches of single nucleotides can be detected in this way to indicate if any of our tumor series may have involved ras structural alterations which could well complement myc and myb expression. These alterations can be worked up by genomic cloning or by using the oligonucleotides for sequencing of ras from those tumors in which mutations can be detected. This study will be extended to other oncogenes with tyrosine kinase activities if ras mutations cannot be implicated. We will also consider other aspects of viral "hit and run" as the second alteration that sped up the tumor development when various viruses were used in the induction.

Different Modes of c-myb Activation and the Role of myb Gene in Tumor Development (Grace Shen-Ong, LG, NCI)

Objectives:

1) To elucidate the modes of disruption of the c-myb oncogene locus due to site specific virus-insertion in 6 out of 6 independently derived Abelson virus-induced plasmacytoid lymphosarcomas (ABPLs).

2) Since the transforming potential of the chicken myb oncogene has been shown to be restricted to myelomonocytic cells, it is of interest to phenotypically characterized the ABPL cell type to see if the target cell specificity is also conserved in the mouse species or if these tumors are of B-cell origin as they were previously thought to be based on their morphology.

3) To determine if the ABPL cells can be induced to terminally differentiate.

Major Findings:

Rearrangement in the c-myb locus in each of 6 independently derived ABPLs is due to the insertion of a defective Moloney murine leukemia virus (M-MuLV) in the same transcriptional orientation into the intron immediately upstream of the 5'-most exon with v-myb homology. cDNA sequence data and S1 mapping studies reveal the mechanism by which an abnormal myb transcript(s) is derived from the virus-disrupted allele. A novel c-myb protein, presumably initiated within the gag sequence of the inserted provirus and truncated at the N-terminus, is found in the ABPLs.

In collaboration with Dr. H.C. Morse, III (LVD, NIAID), we have established clonal cell lines from different ABPLs and found that they are not in the lymphocyte differentiation lineage, but express various characteristics of myelomonocytic cells. Cloning studies showed that stable lines with phenotypes of different maturation stages could be obtained and passaged continuously in vitro without significantly changing phenotypes. However, treatment of these cells with phorbol-12-myristic-13-acetate (PMA) results in the induction of varying degrees of myelomonocytic differentiation. We selected three of the

APBL lines with different degrees of maturity and studied their responses to PMA treatment. All three lines responded rapidly to PMA, possibly via the activation of protein kinase C, but differed in the extent they could differentiate into macrophage-like cells. Single cell cloning experiments showed that clones cannot be obtained in the presence of PMA from the most mature ABPL lines which fully differentiate into macrophage-like cells, whereas cloning efficiencies were equivalent in the presence or absence of PMA for cell lines with the less mature phenotypes and only a limited capacity to differentiate upon PMA treatment. Hence, the more mature ABPL cells can terminally differentiate under in vitro limiting-dilution conditions. However, continuous exposure of the more mature cell lines to PMA in bulk cultures results in a marked slowing of cell growth rather than cell death, indicating other features of these culture conditions might be important to the maintenance of cell viability.

We proceeded to examine the influence of virus insertion and the effect of PMA on myb expression in the cells. We found that myb expression was predominantly from the virus-disrupted c-myb locus in the ABPL cells, and the virally-promoted myb RNA levels increased drastically after PMA treatment even if the cells were induced to differentiate. This is in contrast to earlier studies on induced differentiation of myelomonocytic cells with normal myb locus which showed substantial reductions in myb expression in differentiated cells that had ceased to proliferate. We have also found that the levels of the virally-promoted myb RNAs correlated well with those of the other long terminal repeat-promoted helper viral RNAs in these cells. It, therefore, appears that not only is the expression of the myb gene no longer under the normal regulation of the myb gene locus, it has come under the control of the inserted viral promoter which remains responsive to other regulatory control(s), resulting in the inappropriate expression of the myb locus during myeloid development.

Significance to Biomedical Research and Program of the Institute:

We have identified the disruption of the myb locus to be a critical common event in ABPLs and have found these tumors to be of the myelomonocytic lineage. This affirms the notion that the transforming potential of the myb gene is restricted to myelomonocytic cells. The phenotypic characteristics of cell lines established from ABPLs resemble closely those of human myelogenous leukemia. Understanding the mechanisms of disruption of the myb locus at the DNA, RNA and protein levels may lead to an understanding of the role of myb gene in myeloid cell devlopment. The availability of cell lines 'frozen' at various stages of maturation with variable abilities to differentiate may also facilitate the dissection of different events and signals that are important in the proliferation and differentiation of myeloid cells.

Proposed Course of Research:

a) To elucidate the contribution of structural changes of the myb protein and/or the changes of the regulation of myb expression to neoplastic development of myeloid cells.

b) To determine if myb could play a role in neoplastic development of myeloid or other cell types by gene transfer experiments.

c) To determine the exact structure of the 5' end of the human c-myb locus. These coding and noncoding exons are upstream to the site of the virus insertion in ABPLs. Preliminary data reveal the presence of myb transcripts with different 5' end, suggesting the importance of this region of the myb gene product.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05553-17 LGN

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

COOPERATING UNITS *(if any)*

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

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

I. Gene evolution. Genes encoding members of a kappa chain subgroup have been cloned from recombinant DNA libraries generated in six different wild mouse species. Sequence analysis of thse genes is currently in progress. Results from these studies will permit an assessment of multigene evolution during modern speciation and provide insights into mutational mechanisms operating on such families.

II. Growth and regulation. A factor required for plasmacytoma growth in vitro has been purified to apparent homogeneity. Experiments are currently in progress to obtain amino terminal sequence data which will be used to generate a synthetic oligonucleotide probe for gene cloning. Attempts are also underway to raise monoclonal antibodies to this factor.

III. Antibody diversity. Light chains from antibodies to β(1,6)-galactan are unusual in that they express an Ile at the point of V_K-J_K recombination. This amino acid cannot be generated by sequences usually found at the 3' end of V_K genes or 5' to J_K genes. To determine whether nucleotides generating this amino acid were germ encoded or added during recombination, the germline gene and cDNA encoding this variable region were cloned and sequenced. Results of these studies confirm that the codon for the unusual Ile is in the germline immediately 3' to the normal termination of the V_K coding sequence.

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

Objectives:

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

II. Control of growth and differentiation. Studies are continuing to characterize and manipulate a macrophage produced factor involved in growth and survival of plasmacytomas in vitro. Additional studies have been initiated to use expression or subtractive cDNA libraries in an attempt to isolate genes involved in the control of B cell differentiation.

III. Antibody diversity. We have previously observed that in light chains from antibodies to β(1,6)-D-galactan unexpected amino acid was found by protein sequence analysis at the point of V_K-J_K recombination. We, therefore, wished to establish whether the nucleotides encoding this amino acids were added without a template during recombination, as is the case in heavy chain V_H-D and D-J_H recombination, or if these nucleotides were encoded in the germline.

IV. Instrumentation facility.

Major Findings:

I. In the previous annual report we described the construction (with support of NCI contract NO1-CB-33934) of six recombinant DNA libraries from wild mouse species in our colony. These libraries were screened with probes corresponding to the immunoglobulin kappa constant region (a single copy gene) as well as a kappa variable region subgroup containing at least four genes in the inbred BALB/c mouse. Sequences and evolutionary patterns for these genes were described for M. pahari, believed to be the most ancient mouse species in existence today. During the past year, work has proceeded on the sequencing of homologous genes from the additional libraries. The coding regions from 6 V_K genes of M. cookii have been completed and similar (albeit slower) progress is being made in the other species. It is anticipated that most of the sequencing will be completed within the next year at which time we will be in a position to begin analysis of multigene family evolution in the context of modern speciation within the genus Mus.

II. a) Plasmacytoma growth factor (PCTGF). The major effort in this project has been carried out by R. Nordan (see attached). In summary, pilot studies have resulted in the purification, to apparent homogeneity, of PCTGF. Large scale purification has followed these experiments and it is anticipated that sufficient material will be available to attempt protein sequence analysis within the next month. Simultaneously, semi-pure material has been used to immunize rats and fusions are currently being performed in an attempt to generate monoclonal antibodies to PCTGF.

As a complementary approach, Joan Hyde has begun construction of a cDNA expression library using poly A selected mRNA from the PCTCF producing P388D1 cell line. The Okiyama-Berg mammalian expression vectors are being used and we anticipate screening this library either for biologic activity, with monoclonal antibodies should they be available, or synthetic oligonucleotides generated from amino acid sequence determination.

b) B cell growth and differentiation. During the past year W. Davidson (see attached) has attempted to induce pre-B cell lines to differentiate into more mature cell forms found in the B cell lineage by use of LPS and other agents. Our intent was to use such lines to generate subtractive DNA libraries to isolate genes regulating programmed growth and differentiation. To date, we have been unable to induce such lines to proceed sufficiently through B cell development to be useful as a model. A number of sublines characterized from such stimulations expressed new B cell markers but none went on to immunoglobulin expression. Furthermore, several other sublines were clearly phenotypically myeloid. Thus, while we have potentially identified a precursor cell which can subsequently enter either the B cell or myeloid lineage, we have been unable to generate the desired model for B cell differentiation.

As an alternative, we are now planning to construct cDNA from a mature B cell line which will first be used in an expression vector to attempt to isolate genes corresponding to known lymphocyte markers such as ThB and Lyb-2 for which monoclonal antibodies are available. Secondly, we will use this cDNA to subtract against message from pre-B cells in an attempt to isolate genes related to this developmental pathway.

III. Antibody diversity. In previous studies we have reported that 15/16 light chains from antibodies to β(1,6)-D-galactan had an Ile at the point of V_K-J_K recombination. This amino acid could not be generated by recombination between nucleotides normally found at the end of κ chain V regions and the beginning of κ chain J regions. Since nucleotides can be added randomly without a template at the sites of heavy chain V_H-D and D-J_H recombination, it was important to know if a similar process operated at the site of light chain recombination to generate diversity in the third hypervariable region. We therefore used a J_K probe to the rearranged gene encoding this light chain. A specific 5' flanking region probe was then made in order to distinguish this gene from other closely related members of the same family. Using the 5' probe, a genomic library was screened and the germline gene encoding the anti-galactan light chains isolated and sequenced. A comparison of the germline and previously determined cDNA sequence revealed that the Ile in question was, in fact, encoded in the germline immediately 3' to the pont at which V region genes normally end. Thus, this V region gene was effectively one codon longer than usually seen and the extra V region codon was always compensated by a deletion of the first codon contributed by J_K to maintain a normal size. The results from these experiments indicate that, in spite of identical recognition sequences and spacing of these sequences in light and heavy chains, random nucleotides are added only during recombination of heavy chain segments and not in the case of light chains.

IV. Operation of research instrumentation facility. Considerable resources from this lab are dedicated to the operation of a protein micro-sequencing facility. This facility is used collaboratively by members of the Laboratory of Genetics as well as numerous other NCI and NIH investigators. We have been exploring the addition of an oligonucleotide synthesizer to this facility for generation of hybridization and sequencing probes. This approach appears quite feasible for nucleotide sequencing as I have determined over 5 Kb in sequence in an extremely short period using synthetic oligonucleotide priming.

Significance to Biomedical Research and the Program of the Institute:

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

II. The identification and purification of a growth factor necessary for plasmacytoma survival in vitro presents the opportunity to potentially assess the role of this molecule in in vivo neoplasia and/or normal growth. The characterization of other molecules associated with stages of growth and/or differentiation similarly provides systems in which the biological roles of these entities can be evaluated. It is the purpose of such studies to define molecularly the role of characterized genes in the control of cell growth and differentiation.

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

Objectives:

In BALB/cAnPt mice, plasmacytomas (PCT) arise exclusively in the granulomatous tissue which forms in response to the intraperitoneal administration of pristane. This suggests that their growth is dependent upon microenvironmental influences provided by this inflammatory environment. In order to identify factors involved in this process, PCT cell lines have been established in this laboratory which are dependent upon one such factor, PCT-GF, for proliferation and survival in vitro. PCT-GF is produced by normal murine spleen cells and peritoneal macrophages, the P388D1 macrophage cell line and normal human peripheral blood monocytes. The studies outlined here address the initial steps which are necessary to evaluate the role of PCT-GF in plasmacytomagenesis and possibly in normal cell growth. The objectives of these studies have been, first, to determine if any known lymphokines or other biological factors are responsible for PCT-GF activity and, second, to characterize PCT-GF from biological, biochemical and molecular biological viewpoints.

Experiments to examine the effect of PCT-GF on cell cycle parameters and the expression of cell surface transferin receptors are underway in collaboration with Dr. Leonard Neckers. In addition, homogeneous PCT-GF will be used to

identify putative PCT-GF receptors and to examine the distribution of the receptor on normal and malignant cell populations.

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

Major Findings:

1. PCT-GF is distinct from other well characterized factors including IL-1, IL-2, IL-3, macrophage-CSF, BSF-1, BCGF II, EGF, TGFβ, γ and β-IFN and thymosin fraction five, none of which are able to support the growth of the factor dependent PCT cell lines.

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

3. P388D1-derived PCT-GF has been purified to apparent homogeneity (single band on silver stained PAGE). It has an apparent size of 24 kDa and apparent isoelectric points of 6.2 and 6.4. We are proceeding with the isolation of PCT-GF to be used in experiments designed to determine a partial amino acid sequence, to clone the gene encoding PCT-GF and to produce antisera and monoclonal antibodies to PCT-GF.

4. Rat antiserum to PCT-GF has been produced by hyperimmunization with liposomes containing partially purified PCT-GF. This antiserum inhibits PCT-GF activity *in vitro* at dilutions up to 1/20,000.

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

Methods Employed:

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

For the purification of PCT-GF, we have developed the following protocol: (1) adsorbtion to and elution from C-4 derivatized controlled-pore glass beads, (2) size exclusion chromatography on Sephadex G75, (3) chromatofocusing (FPLC) and (4) reversed phase HPLC. Using this protocol we have obtained approximately 300 ng of homogeneous PCT-GF from 5 liters of P388D1 supernatant. A preparative isolation is underway, and we expect to have enough purified material for a partial amino acid sequence within one month.

For the production of antiserum and monoclonal antibodies, we have immunized rats i.v. with sub-microgram amounts of partially purified PCT-GF encapulated in liposomes containing lipid A. An assay which detects inhibitory αPCT-GF antibodies has been developed. A highly inhibitory rat antiserum has been obtained, and we are attempting to produce monoclonal antibody-producing hybridomas. Highly specific rabbit antiserum to homogeneous PCT-GF will be produced using the liposome adjuvants.

Significance to Biomedical Research and the Program of the Institute:

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

Proposed Course of Research:

1. We plan to proceed with the preparative isolation of PCT-GF to determine a partial amino sequence of PCT-GF and to clone the gene encoding PCT-GF.

2. To produce rat monoclonal antibodies and highly specific rabbit anti-serum to examine (1) the role of elevated PCT-GF levels in plasmacytomagenesis and (2) the role of PCT-GF in normal B lymphocyte development.

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

Publications:

Rudikoff, S.: Somatic diversity and mutation. In Kelsoe, G. and Schulze, D. (eds.) Evolution and Vertebrate Immunity: The antigen-receptor and MHC gene families, in press.

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In Potter, M., Nadeau, J.H. and Cancro, M.J. (eds.) Current Topics in Microbiology and Immunology, Vol. 127., Heidelberg, Springer-Verlag, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08726-09 LGN

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Biochemistry and molecular biology of transplantation antigens

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

P. I.: Michael J. Rogers	Research Chemist	LGN,NCI
Richard Swerdlow	Staff Fellow	LGN,NCI
David Siwarski	Bio. Lb. Tech.	LGN,NCI
Saswati Chatterjee-Das	Guest Worker	LGN,NCI
Lloyd Law	Chief, Lab. of Cell Biology	LCBGY,NCI

COOPERATING UNITS *(if any)* Dr. John Coligan, NIAID; Dr. Giorgio Parmiani, National Cancer Institute, Milan, Italy; Stuart Rudikoff, LGN,NCI; Dr. Stanley Nathenson, Albert Einstein College of Med., NY; Dr. Richard Flavell, Biogen Research Corp.; Dr. Linda Gooding, Emery Univ., Atlanta, GA

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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

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

In the case of H-2 antigens, the approach is to identify class I molecules expressed by tumor cells and determine which of the many class I genes encode them. Alloantisera, monoclonal antibodies and xenoantisera directed against H-2 molecules are used to purify and characterize the proteins. Specific DNA probes and molecular cloning techniques are then used to identify the mRNA and genes that encode these H-2 molecules. This work is aimed toward understanding any special role that H-2 molecules might have in tumor cell biology and anti-tumor immunity. The results also contribute to an understanding of the organization and evolution of the class I multigene family.

GPO 914-918

Project Description

A. Tumor Associated Transplantation Antigens

Objectives:

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

Major Findings and Proposed Course of Research:

Gp175. We recently identified and purified a 175,000 dalton cell surface glycoprotein from RBL-5 (gp175) which bears a TATA that not only protects against challenge with RBL-5 but also another tumor induced by murine leukemia virus. Dr. Swerdlow has been characterizing this molecule in an attempt to understand the molecular basis of its immunogenicity and its role in normal cellular metabolism. These questions are especially intriguing since gp175 is expressed by all murine cells that we have so far tested, but it is immunogenic only when expressed by RBL-5.

Dr. Swerdlow has found that, although gp175 is clearly a glycoprotein, it contains a relatively small amount of sugar compared to other cell surface glycoproteins with molecular weights near 150,000 daltons. These other proteins usually contain 10-30% carbohydrate by weight. In contrast, gp175 contains only 2-3% N-linked carbohydrate and there is no evidence for O-linked carbohydrate. This finding led us to believe that the antigenic structure of gp175 was part of the polypeptide chain. Accordingly, Dr. Swerdlow has now refined the purification protocol to yield sufficient amounts of gp175 for direct sequence analysis. Metabolically labeled gp175 was first purified by immunoprecipitation and elution from polyacrylamide gels. This labeled material yielded a sequence thereby establishing that the N-terminus was not blocked. Approximately 500 µg of gp175 has now been purified and is currently being sequenced in collaboration with Dr. S. Rudikoff.

TATA Heterogeneity. Dr. G. Galetto completed his interesting analysis of the number of TATAs expressed by tumor cells and their relative contribution to anti-tumor immunity. We demonstrated that RBL-5 expressed a minimum of one additional glycoprotein that was at least as immunogenic as gp175. Similar results have been obtained with other tumors. In an effort to determine the relative immunogenicity of these multiple TATAs, we used affinity chromatography with rabbit anti TATA immunoabsorbants to specifically and quantitatively remove TATA from whole cell lysates of RBL-5 cells. In order to generalize our experiments we also removed TATAs from two unrelated tumors, Meth A and mKSA, in the same way. To our surprise, in each case removal of these TATAs had virtually no effect on the immunogenicity of the whole cell lysate. These results suggest that purification

of a TATA by selecting the most immunogenic fractions will generally not yield the most immunogenic TATA. The implication of these results is that a single, dominant, immunogenic structure does not exist on tumor cells. Instead, the anti-tumor immune response is simultaneously directed toward many antigens. Purification yields the most chemically stable and/or abundant molecule, not necessarily the most immunogenic.

Future Work

We hope that the amino acid sequence of gp175 will provide clues to the identity of this protein and the basis of its immunogenecity. That failing, DNA probes can be constructd on the basis of this sequence and used to continue this problem at the genetic level. Dr. Galetto's studies indicated to us that purification of immunogenic proteins from tumor cells, while providing potentially interesting information about a particular TATA, was not the best approach to understanding the overall nature of the target of the anti-tumor immune response. Accordingly, we have begun a broader genetic approach to this problem which will be the focus of our efforts in the near future.

This approach takes advantage of the fact that molecules bearing the TATA of the chemically induced tumor Meth A, have been mapped to a 12:X translocated chromosome. This translocated chromosome has been isolated in a mouse-hamster hybrid cell line and shown to express a TATA which is indistinguishable from the Meth A TATA in all respects.

Dr. Swerdlow is now isolating DNA probes that detect transcripts from this 12:X translocated chromosome. DNA from the mouse-hamster hybrid cell is digested with a restriction endonuclease and converted to single stranded form. Single stranded DNA specifically hybridizing to Meth A mRNA is then selected and cloned into an appropriate vector. Clones which bind only to Meth A mRNA and not to normal fibroblast mRNA are then selected by Northern blotting. These clones are used to screen a complete Meth A cDNA library. The cDNA clones isolated in this way should be enriched for tumor specific mRNAs that have been transcribed from the translocated 12:X chromosome. We hope that analysis of these clones will yield information regarding a full spectrum of the molecules that encode the Meth A TATA.

B. Histocompatibility Antigens

Objectives:

The goal of this work is to understand the mechanisms which control the expression of the many class I genes present in the murine genome, with particular emphasis on those class I genes expressed on tumor cells. There are some indications that tumor cells may express inappropriate class I molecules that contribute to their biological and immunological properties and we would like to obtain direct evidence for this phenomena. In addition, the process of tumorigenesis may "trap" cells in particular stages of differentiation during which certain class I molecules are normally only transiently expressed. This stable expression in the tumor could reveal a rare class I transcript that would otherwise go undetected.

Major Findings and Proposed Course of Research:

H-2 molecules on tumor cells.

We have been continuing an analysis of the chemically induced B-cell lymphoma of SJL/J origin, DMLM1678. We originally chose this tumor because it was lysed by several inappropriate alloantisera suggesting that it expressed unusual class I molecules. We were able to show that this tumor expresses one additional class I protein besides the normal H-2K^s and H-2D^s proteins. The gene encoding this protein mapped to the right of S suggesting that it might be in the Qa region.

In collaboration with Dr. John Coligan's group we have now shown that there are actually three different molecules precipitated from tumor cells with anti H-2K^s alloantiserum. We have determined the partial N-terminal sequences of all three proteins and compared their tryptic peptide maps. In addition we have determined which proteins are phosphorylated and which bind to a rabbit antisera prepared against a synthetic peptide corresponding to the canonical C-terminal sequence of the H-2K^b molecule. The results have allowed us to conclude that all three H-2K^s proteins are encoded by the same class I gene and are generated by alternative splicing of pre-mRNA. This is the first report of three class I proteins being encoded by a single class I gene.

We are now preparing a cDNA library from this tumor. We plan to isolate all class I cDNAs from this library and look for the 5 species predicted by our protein studies as well as any others that may be present.

Evolution of class I genes

The wild mouse colony maintained by Dr. M. Potter provides a unique source of genetic material. Consequently, in conjunction with our studies on class I protein expression, we have been analyzing the class I gene complement in species of Mus distantly related to the inbred strains. The immediate goal of this study is to analyze the expansion and contraction of the class I gene family. Dynamic changes in the size of the class I gene family are evident from comparisons of gene number in mouse, man and swine. Our initial studies suggested that expansions and contractions also occurred over the shorter periods of evolutionary time (~ 10^7 years) represented by the species in Dr. Potter's colony.

We have continued our studies by cloning several class I genes from the species Mus pahari. This murid diverged from the Mus domesticus line (inbred mice) about 8-11 million years ago. Two phage clones containing class I genes have been isolated from a genomic library prepared from liver DNA of this species. The first clone contains a single, complete class I gene which has been almost entirely sequenced. The second contains a complete gene and a partial gene. The sequences of both of these genes have also been determined. Flanking region probes and coding region probes from these genes have been prepared as well. These probes as well as single copy probes for other class I genes obtained from various sources are being used to determine the copy number of

several class I genes in the genomes of all of the species in Dr. Potter's colony. Some of this work is being done in collaboration with Dr. Richard Flavell and Dr. Stanley Nathenson.

So far, these studies have provided evidence for the following: 1. The Q10 gene has undergone dramatic expansions during relatively short periods of evolutionary time. 2. The T1-TL3 gene pair has undergone expansion and contraction independently during the same period of time and has been completely deleted from some species. 3. The partial gene in our Mus pahari clone seems to be the remnant of an unequal crossing over event which is the type of DNA rearrangement that could lead to the changes in gene number that we are seeing. 4. The overall exon-intron structure and DNA sequence of these genes from Mus pahari has confirmed many of the conclusions about evolution drawn from comparing class I genes from inbred mice. In addition, highly conserved, non-coding regions have been identified which may be potential control elements.

We plan to complete the sequencing of the two genes that we have already cloned. Future work will also involve seeking more information about the dynamic size changes of this gene family by probing our unique collection of DNAs from the entire Mus genera with other single copy class I DNA probes. Eventually, we hope to construct a cosmid library from Mus pahari to obtain detailed information about the overall size and organization of the MHC in this species.

Significance to Biomedical Research and the Program of the Institute:

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

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

Publications:

Galetto, G., Law, L.W., and Rogers, M.J.: The Rauscher MuLV induced leukemia RBL-5, bears two tumor-associated transplantation antigens expressed on distinct molecules. Int. J. Cancer 36: 713-719, 1985.

Rogers, M.J., Siwarski, D.F., Jouvin-Marche, E., and Rudikoff, S.: Gene specific structures within class I genes from Mus musculus domesticus are conserved in class I genes from Mus pahari. In Potter, M., Nadeau, J.H. and Cancro, M.J. (eds.), Current Topics in Microbiology and Immunology, Vol. 127, Heidelberg, Springer-Verlag, in press.

Rogers, M.J., Siwarski, D.F., Shacter, E., Maloy, W.L., Lillehoj, E.P., and Coligan, J.E.: Three distinct H-2K^{s} molecules differing at the carboxy terminus are expressed on a tumor from SJL/J mice. J. Immunol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05552-17 LGN

PERIOD COVERED

October 1, 1985 to September 30, 1986

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. Nisini	Visiting Scientist	LGN,NCI

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LAB/BRANCH

Laboratory of Genetics

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☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece 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 involving 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. Ambesi, 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 clones from 3rd to 6th passage cultures. We have initiated a hybridoma screen using OLFE as antigen and fluorescent anti-mouse IgG staining of frozen sections of OLFE for selection. These results, too, are very encouraging; many of the hybridoma supernates appear to identify small patches of cells selectively in the OLFE. The sensory neurons of the olfactory epithelium are renewed throughout life from a stem cell population. Development of this system would make available the first mammalian neuroblast to neuron cell culture system. It is hoped that basic issues in olfactory sensory physiology can be explored with this system.

I. Biology of the Mammalian Olfactory Epithelium

Objectives:

The mammalian olfactory epithelium (OLFE) represents a timely challenge to cell biologists, neurobiologists and cell culturists. The cellular mechanism for the chemoreception in the OLFE is not understood. Presumably, there are receptors in the dentritic tree of the sensory cells, but how many types of receptors? how many receptors per cell? A particularly intriguing feature is that these sensory neurons have half-lives of 30 days in mouse and man and are, therefore, constantly being renewed from a stem cell population. The organ is planar, a pseudostratified epithelium, and therefore well suited for bringing into cell culture, where our goals would be to clone and compare receptor activity among clones. The development of clonal cell strains with characteristic responses to different oderants would be a great advance in the attempt to understand both neuroblasts in general and to understand olfactory receptor biology in particular. The history of past attempts implies that it will be crucial to success to culture the whole system of OLFE cells (at least 3 cell types) in order to find conditions for normal functions to be expressed in vitro.

The epithelium should also be studied in situ or after the cribriform plate and proximal turbinates are removed (from neonatal rat pups). These preparations could be examined using voltage sensitive fluorescent dyes, fluorescent or radioactive oderants in the hope of identifying differential patterns of activity in cells from various regions of the OLFE and how such patterns might change during challenge by different oderants. A hybridoma screen has been undertaken in the hope of finding antibodies that recognize specific cell types in the OLFE. It might even be possible to obtain antisera directed against specific receptors and thus have entree directly into the molecular biology of the receptor gene family. The goals of this work then are primarily to develop a suitable cell system (presumably clonal cultures of the sensory neurons' stem cell) for the elucidation of the basic biology: the cellular distribution of oderant receptors and their ultimate molecular characterization and molecular genetics. The receptors are the key objective of the study; the cell system is a means toward that goal and perhaps others of importance as well.

Methods Employed:

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

The strategy we have used has been to initiate cultures of 1 or 2 day post partum Fisher rat OLFE fragments on or in basement membrane gels. These primary tissue cultures served as the basis for assaying various complex medium formulations and were undoubtedly responsible for our ultimate success where many others had failed to get good growth with dissociated primary cells. When cells grow out into or onto the gel, one can observe the effect of different medium additives with minimal trauma to the cells. The first successful medium we have developed contains: hypothalamus extract, pituitary extract, fetal calf serum, insulin, transferrin, selenous acid, hydrocortisone, tri-iodothyronine, retinoic acid, and galactose, in a .1 mM Ca^{++}, .4 mM Mg^{++} Coon's modified F12 base. The cells grow best (and perhaps the ones we are interested in most grow only) in plates that have been coated with the basement membrane preparation.

Major Findings:

We have developed a medium and culture conditions that permit continuous growth of several cell types from the neonatal rat OLFE. Using the mixed cultures thus obtained, conditioned medium has been found to support clonal growth of several distinctive cell morphologies. These are being evaluated.

We have found that when the cells are growing on sparcely coated regions of the plates, they divide rapidly and look like fibroblasts. When, however, the same cells are seeded on thick, more dilute gels, they aggregate into clusters of 10 to 50 cells and appear to differentiate. They are bipolar and are all oriented in the same radial pattern with a blunt, often bulbous end directed outward and a single long, fine, branched axon-like projection at the basal pole. The axon-like projections are often seen associated with cells that appear to be Schwann's cells in the same cultures. The more blunt, outwardly directed pole often produces a few (up to ten per cell) fine, isodiametric projections like filopodia or cilia. Little or no division has been seen in these cell clusters. It is possible that the differentiation of the sensory cells in culture will not be difficult to achieve.

Proposed Course of Research:

We expect to continue as we have done improving the culture techniques. The cloning procedures are especially important. Our main task is to try to characterize the cells that we have cloned and that are growing in mass culture. Thus far, we have not demonstrated olfactory marker protein (OMP) in these cells unequivocally. That protein, of unknown function, is known to be present in the cytosol of olfactory sensory neurons (as well as some cells in the olfactory bulb). OMP, however, is thought to accumulate only in cells that have made synaptic contact. We do not now include a source of olfactory bulb mitral cells in our cultures. We may be able to trick the cells into believing that they have synaptic relations by adding large amounts of the transmitter (carnosine). It may not be necessary to add mitral cells or any transmitter to get expression of the receptors which are our main interest. I believe that the best authentication of these cells will come from electrophysiological measurement of changes in membrane potential (depolarization currents) as a consequence of micro-perfusion or oderants. We shall attempt in the near future to set up such measurements and to develop the needed collaboration.

In the end, the most important part of the work could turn out to be the hybridoma screen. We are using a boost schedule of 3, 4 and 5 times, three weeks apart. The hope is to increase among the hybridomas activity against the more rare antigens by going out to long immunization schedules. This work, though labor intensive, is relatively cheap, and we shall continue to pursue it vigorously.

Significance to Biomedical Research and the Program of the Institute:

As noted above there is little cell-level knowledge of the physiology and no biochemistry of the sensory cells of the OLFE. The OLFE is almost virgin territory. Successful culture of these cells promises a meaningful advance in the study of (neuro)blast → neuron differentiation as well as various intriguing aspects of sensory coding in a system that may show specific mapping in a way similar to the retino-tectal system. The mystery inherent in the challenge of recognizing as many as 10,000 different oderants poses unique problems for receptor biology perhaps not unlike those posed for the immune system a decade ago. The importance to sensory neurobiology of a culture system like this cannot be overstated. There are only a few cell systems in culture that appear to show blast cell → differentiated cell(s) maturation *in vitro*. None of these are neurons, let alone specific sensory neurons. The role of oncogenes and other endogenous growth vs. differentiation substances could well be studied in culture systems like this one.

Publications:

Brandi, M.L., Fitzpatrick, L.A., Coon, H.G., and Aurbach, G.D.: Bovine parathyroid cells: Cultures maintained for more than 140 population doublings. *Proc. Natl. Acad. Sci. USA* 83: 1709-1713, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08950-04 LGN

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

P.I.:	Sandra Smith-Gill	Sr. Staff Fellow	LGN,NCI
	C. Mainhart	Microbiologist	LGN,NCI
	T. B. Lavoie	Biologist	LGN,NCI
	E. Vacchio	Biologist	LGN,NCI
	R. Duncan	Biologist	LGN,NCI
	C. Mallett	American Cancer Society Fellow	LGN,NCI

COOPERATING UNITS *(if any)*
W. Drohan, Molecular Genetics Group, Meloy Laboratories, VA; K. Dorrington, Dept. of Biochemistry, University of Toronto, Toronto, Canada; D. Davies, LMB, NIADKD; B. Brookes, DCRT, NIH

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☐ (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, are used as probes to study antibody-protein interactions and structure-function relationships and to study developmentally regulated antigens in normal and neoplastic development. The interaction of antibodies with the epitopes are modelled and ultimately refined by protein crystallography studies. One antibody has been studied in detail; the computer refined combining site is a shallow concavity bordered by hydrophobic segments. The computer fitted complex of this antibody with the proposed epitope in lysozyme indicates a very extensive contact interface involving antibody framework residues as well as 5 out of 6 complementarity determining regions. The opposing surfaces of the antibody and antigen are highly complementary in shape, charge, and hydrophobic properties, and side chain-to-backbone hydrogen bonds predominate in the interaction between antibody and antigen. Chain recombination experiments have allowed further definition of likely contact residues with HEL and have in addition provided evidence for a hierarchy of structure-function determination. Experiments to test hypothesized structure-function correlates utilizing site specific mutagenesis of in vitro expressed immunoglobulin genes are in progress. Monoclonal antibodies are being 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

In order to understand the molecular basis of interaction between an antibody and a protein antigen, we have concentrated on solving the structures of high affinity monoclonal antibodies (mAbs) specific for well-defined epitopes on the model protein antigen, hen egg white lysozyme (HEL), and of the complexes of these antibodies with HEL. In addition, C. Mallett has been producing a panel of lower affinity HEL-binding primary-response and "Natural" mAbs that will be compared with the high affinity, high specificity mAbs.

The heavy and light chain V-region sequences of 4 anti-HEL mAbs (HyHEL-5,-8, -9,-10) and a related hapten-binding myeloma protein (XRPC-25) have been determined from cDNA clones by T. Lavoie, in collaboration with Dr. W. Drohan, Meloy Laboratories, and by primer extension of mRNA, in collaboration with K. Huppi. C. Mainhart has modelled and energy minimized the three-dimensional structure of HyHEL-10 Fv, in collaboration with B. Brooks (DCRT). Notable features of the combining site include: (1) the combining region is an acidic, hydrophillic, shallow concavity, approximately 20 x 25 A, bordered by hydrophobic segments; (2) variation in length of the complementarity-determining regions may function not only to directly change the shape of the antibody combining site, but may also indirectly influence the nature of the antibody surface by changing the accessibility of residues not usually involved in antigen binding (framework residue 49L in HyHEL-10). We have computer fitted the HyHEL-10 Fv to the proposed epitope on HEL, and we have developed a new methodology for systematically exploring the interaction between two molecules. This fitting indicates that the contact surface is very extensive, at least 400 A^2, involving 5 out of 6 CDR regions as well as framework residues of the antibody. The opposing surfaces of the antibody and antigen are highly complementary in shape, charge, and hydrophobic properties. Side chain-to-backbone hydrogen bonds predominate the interaction between antibody and antigen, and are likely an important component of antibody-protein interactions in general. D. Davies' laboratory (LMB, NIADKD) are currently analysing HyHEL-10 and HyHEL-5 Fab-HEL complexes by X-ray crystallography at 3.0 and 2.0 A resolution, respectively.

Chain recombination experiments, performed in collaboration with K. Dorrington, Toronto University, have allowed further definition of likely contact residues with HEL, and have demonstrated that the ability to bind HEL versus several haptens is absolutely heavy chain determined, while H and L chains interact to determine fine specificity and affinity. The results also provide experimental evidence for a hierarchy of structure-function determination: combinational diversity can confer unique, nonoverlapping specificity to the same V_LV_H pair; a given assembled V_LV_H can support binding of multiple specificities when combined with different V_H or V_L, respectively; and somatic mutation can function to alter patterns of fine specificity and crossreactivity, independent of affinity, but not overall specificity.

In order to test hypothesized structure-function relationships, T. Lavoie has cloned the rearranged cDNA and genomic DNA clones of HyHEL-8,-10, and XRPC-25 constructed a vector for expression of immunoglobulin genes in eukaryotic cells and to date has successfully expressed the HyHEL-10 L chain in SP2/0 cells.

Altered HyHEL-10 sequences have been produced by oligonucleotide-directed mutagenesis in M13 with emphasis on the role of the H chain, specifically H3, in determining HEL specificity.

The structure-function relationships emerging from our studies with a model protein system can now be applied to immunochemical analysis of the antigenic and functional structure of an undefined oncogene protein product, mouse c-myc. E. Vacchio has obtained rabbit antisera to bacterially expressed myc protein in collaboration with F. Mushinski. This antisera will be used for purification of larger quantities of myc protein and to study the function and subcellular localization of the native myc protein in mouse plasmacytomas. Monoclonal mAbs to myc protein will be utilized to probe structure and function of myc. Recombinant DNA technology will be utilized to produce variant myc protein products for use in epitope mapping and for dissection of structure-function relationships.

Experiments are in progress to develop tetraparental mouse embryos utilizing tumor-resistant and tumor-susceptible strains. The resulting chimeric mice will be utilized in tumor-induction experiments to examine the cell-specificity of resistance genes in plasmacytoma development.

Publications:

Smith-Gill, S.J., Finkelman, F.D., and Potter, M.: Plasmacytomas and murine immunoglobulins. In Colowick, S.P. and Kaplan, N.O. (eds.): Methods in Enzymology, Vol. 116, Academic Press, New York, pp. 121-145, 1985.

Carper, D., Smith-Gill, S.J., and Kinoshita, J.H.: Immunocytochemical localization of the 27Kβ-crystallin polypeptide in the mouse lens during development using a specific monoclonal antibody: Implications for cataract formation in the Philly mouse. Dev. Biol. 113: 104-109, 1986.

Smith-Gill, S.J., Hamel, P.A., Klein, M.H., Rudikoff, S., and Dorrington, K.: Contribution of the V_K4 light chain to antibody specificity for lysozyme and β(1,6)D-galactan. Mol. Immunol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08951-04 LGN

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular basis for the acute erythroleukemias induced by murine retroviruses

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

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

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☐ (a1) Minors
☐ (a2) Interviews

B

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

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

Investigations on the acute erythroleukemia-inducing virus, spleen focus-forming virus (SFFV), have concentrated on determining which viral sequences are responsible for pathogenicity and erythroid cell specificity. Our data have shown that sequences in the long terminal repeat (LTR) region of SFFV do not determine the erythroid cell tropism of the virus and that expression of the SFFV env gene in the absence of other SFFV genes and the SFFV LTR is pathogenic. Thus, the primary effect of SFFV on erythroid cells, which is to alter the requirement that the cells have for the erythroid hormone erythropoietin, is due to the product of its env gene. To further understand how the viral env gene product may do this, we have been studying variants of the virus, $SFFV_P$ and $SFFV_A$, which both induce acute erythroleukemia in mice but differ in their effects on erythroid cells as well as in the post-translational modification of their env gene products. By making recombinants between $SFFV_P$ and $SFFV_A$, we have been able to localize the sequences responsible for the biological and biochemical differences between these two viruses to a 678 bp region in the 3' region of the envelope gene.

Studies on the genetics of susceptibility to early erythroleukemia induced by Friend murine leukemia virus are concentrating on a gene previously identified on chromosome 5 that encodes a viral envelope protein related to that of mink cell focus-inducing virus. Attempts are being made to confer resistance to leukemia by genetic transfer of this gene as well as to apply this mouse model for resistance to the treatment of human diseases such as AIDS.

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

Major findings:

(1) Sequences in spleen focus-forming virus that determine its tropism for erythroid cells reside outside of the long terminal repeat region.

Although it was well established that the envelope gene of the spleen focus-forming virus (SFFV) is required for the pathogenic effect of the virus, it was unknown whether specific sequences in the long terminal repeat (LTR) were necessary for proper expression of this gene in erythroid target cells. In order to determine whether LTR sequences of SFFV, like those of other murine leukemia viruses, are uniquely required to confer tissue specificity to the virus, we prepared recombinant SFFVs in which the LTR region containing promoter and enhancer functions was replaced with analogous LTR regions from Friend and Moloney ecotropic and mink cell focus-forming viruses. It was found that all of the SFFV constructs, even those with a LTR derived from lymphoma-inducing viruses such as Moloney MuLV, transformed erythroid cells *in vitro* and induced exclusively an erythroid disease. These results demonstrate that sequences in SFFV that determine the tissue-specific nature of the disease reside outside the LTR.

(2) The SFFV env gene, when introduced into mice in the absence of other SFFV genes, induces an acute erythroleukemia.

Previous experiments in our laboratory and others have been consistent with the hypothesis that the SFFV *env* gene is the only SFFV gene essential for the induction of acute erythroleukemia. No studies, however, have been carried out with the SFFV *env* gene in the complete absence of any other SFFV sequences. To accomplish this goal, we isolated only the sequences that encode the envelope glycoprotein of the $SFFV_A$ strain of SFFV and expressed them in a Moloney MuLV-based retrovirus vector. The SFFV sequences were placed in the vector DNA in the the reverse orientation as well as in the proper orientation to provide an experimental control. The vector, which was constructed from 2 clones from Dr. Eli Gilboa, Princeton University, includes a neomycin resistance gene at its 5' end which was used both for selecting cells containing the virus and for titering the virus produced by fibroblasts. When this vector was introduced along with helper virus DNA into fibroblasts, the resulting virus complex induced a rapid erythroid disease that was indistinguishable from that induced by the entire SFFV genome. Spleens from the diseased mice were shown to express the SFFV *env* gene product, but not the SFFV *gag* gene product. As expected, mice given the virus containing the SFFV *env* gene in the reverse orientation did not express the SFFV *env* gene or develop erythroleukemia. These results prove that expression of the SFFV *env* gene in the absence of other SFFV genes and the SFFV LTR is pathogenic.

A side project has also evolved in the course of our efforts to prepare a pathogenic virus from the SFFV *env* gene vector DNA. Our results indicate that certain procedures for preparing viruses from the vector are better than others in generating a virus that expresses high levels of the SFFV envelope protein. We have found that transfection of helper virus-containing cells with the SFFV vector followed by selection of the cells in media containing the neomycin analogue, G418, results in suppression of the SFFV *env* gene. Using this pro-

cedure, we could find no evidence of expression of the SFFV envelope protein in the transfected cells and the released virus caused disease in only a small percentage of mice after a latency of greater than 3 months. Alternatively, when the SFFV vector DNA was co-transfected along with helper virus DNA, which allowed the vector to spread through the culture by the normal process, the cells were shown to express the envelope glycoprotein of SFFV and the released virus caused an erythroleukemia in 100% of the mice after 2-3 weeks. Subsequent selection of vector-containing cells with G418 had no effect on the expression of the SFFV envelope gene. We are now carrying out studies using DNA containing another selectable marker, gpt. Internal suppression of the SFFV env gene should not occur and a higher titer of virus than that obtained by co-transfection with helper virus should be obtained. In addition, the neo resistance gene can still be employed in titering the virus.

(3) Sequences responsible for the biological and biochemical differences between variants of SFFV can be localized to a 678 bp region at the 3' end of the envelope gene.

Two distinct strains of Friend spleen focus-forming virus, $SFFV_P$ and $SFFV_A$, induce a rapid erythroleukemia in susceptible animals. However, $SFFV_P$-infected cells can proliferate and differentiate without an exogenous supply of the erythroid hormone erythropoietin (Epo), while the $SFFV_A$-infected cells still require 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. We have recently shown that a recombinant virus which contains the 3' half of the $SFFV_P$ env gene and the $SFFV_P$ long terminal repeat (LTR) sequences on an $SFFV_A$ background encodes an envelope protein that is post-translationally modified like that of $SFFV_P$ and is capable of inducing all of the biological effects previously associated with $SFFV_P$. In order to rule out the possible role of the $SFFV_P$ LTR, a new recombinant virus was constructed such that the 678 bp EcoRI to ClaI fragment from the 3' end of the env gene of $SFFV_A$ was replaced by the corresponding sequence from $SFFV_P$. This new recombinant virus possesses all of the biological as well as biochemical characteristics of $SFFV_P$. Results from erythroid colony assays indicated that infected cells can differentiate without added Epo and high proliferation was also measured in the absence of Epo in a ^3H-thymidine assay. Further studies indicated that the envelope glycoprotein encoded by this recombinant virus was properly glycosylated to the 65,000 molecular weight form that eventually appears on the cell surface. All of these are unique features of $SFFV_P$-infected cells. Our data, therefore, provide direct evidence that the 678 bp fragment at the 3' end of the $SFFV_P$ env gene is responsible for the biological and biochemical differences between the two viral strains.

(4) Employment of a ^3H-thymidine incorporation assay to distinguish the effects of different Friend erythroleukemia-inducing retroviruses on erythroid cell proliferation.

Spleen cells taken from mice infected as adults with two different variants of the spleen focus-forming virus, $SFFV_P$ and $SFFV_A$, as well as spleen cells taken from mice infected as newborns with Friend MuLV were assayed in a proliferation assay in the presence or absence of the erythroid hormone erythro-

poietin (Epo). Infection of mice with SFFV results in excessive proliferation of erythroid cells that can still differentiate, and spleen cells taken from these mice were able to incorporate high levels of ^{3}H-thymidine in the absence of Epo, even in the presence of antibodies to Epo. The level of proliferation of spleen cells from $SFFV_A$-infected mice, but not those from $SFFV_P$-infected mice, could be greatly enhanced by the addition of Epo to the cultures. Infection of newborn mice with F-MuLV results in the generation of Fr-MCF virus which causes excessive proliferation of erythroid cells. These cells appear to be blocked in differentiation, resulting in severe anemia. Spleen cells from these mice were unable to proliferate in the absence of Epo. However, when increasing doses of Epo were added to the cultures, the cells proliferated at levels equivalent to the levels seen with SFFV. These results indicate that a proliferation assay based upon the incorporation of ^{3}H-thymidine into spleen cells in response to Epo can be used as a quantitative means of assessing and comparing the effects of erythroleukemia-inducing retroviruses on the proliferation of their target cells.

(5) Helper virus is not required for induction of the autonomous stage of transformation in erythroid cells infected with SFFV.

Friend virus complex, which is composed of the spleen focus-forming virus (SFFV) and Friend MuLV, induces in mice an erythroleukemia that has two clearly discernable oncogenic stages. In the first stage, virus infection of a multiple number of erythroid precursor cells results in a polyclonal erythroblastosis characterized by an enlarged spleen and liver. In the second stage, which is apparent later in the course of disease, a population of highly malignant cells can be detercted that have the capacity to be transplanted subcutaneously or into the omentum of syngeneic mice and to grow continuously as cell lines in vitro. Although erythroleukemia cell lines can be established from mice infected with the Friend virus complex, consisting of both F-MuLV and SFFV, the role of SFFV in the development of these malignant lines is unclear. In addition, similar lines can also be established from the spleens of mice infected with F-MuLV alone. In order to determine the role of SFFV in the development of erythroleukemia cell lines, we utilized a previously described helper-free SFFV which induces a rapid erythroid disease having all of the characteristics of that induced by helper-containing stocks of SFFV. Either directly or after transplantation in syngeneic mice, we were able to detect tumorigenic cells in the omentums of both helper-free virus-infected BALB/c and NFS mice. In addition, these cells were able to be propagated in vitro in Dulbecco's modified Eagle's medium with 10% fetal calf serum. These erythroleukemia cell lines, designated MEL-NP, were further analyzed and shown to (1) grow as subcutaneous tumors in syngeneic mice; (2) be free of infectious virus; (3) express SFFV but not F-MuLV viral proteins, (4) express the transformation-related protein p53; and (5) be induced to differentiate into hemoglobin-containing cells using DMSO and HMBA but not Epo. It is, therefore, clear from these studies that replication competent helper virus is not required for the development of the second stage of SFFV-induced transformation. Since SFFV alone can induce both first and second stage erythroid disease and the only virus component present in cells from the different stages is SFFV, one must conclude that the virus is either directly or indirectly responsible for induction of both phases. The availability of transformed erythroid lines

containing only SFFV should facilitate studies of virus integration and the role of the viral genome in malignant transformation as well as provide a source of erythroid cells that can easily be infected with retroviral vectors containing genes involved in erythroid cell differentiation.

(6) Attempts to transfer resistance from congenic mice carrying the $Rmcf^r$ locus to $Rmcf^s$ mice using bone marrow cells.

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. Studies have been undertaken to determine whether resistance can be transferred via the bone marrow from resistant to susceptible mice. Since it is necessary to carry out bone marrow transplants in adult mice and since adult mice are resistant to F-MuLV-induced erythroleukemia, we established another test system for resistance that enabled us to use adult mice. This was based on the knowledge that the $Rmcf^r$ gene controls the replication of Friend MCF virus. Thus, a Friend MCF virus pseudotype of SFFV was prepared and tested. This virus was shown to cause a rapid erythroleukemia in $Rmcf^s$ mice such as NIH Swiss and BALB/c but failed to cause disease in $Rmcf^r$ mice such as DBA/2 or the $Rmcf^r$ C D_2 congenic mouse. Bone marrow cells have now been transferred from BALB/c or C D_2 $Rmcf^r$ mice to irradiatiated BALB/c mice and after 4 weeks the mice will be challenged with FrMCF/SFFV. The results should indicate whether resistance can be transferred via the bone marrow from resistant to susceptible mice. This work is being done in preparation for future studies when the $Rmcf^r$ gene is molecularly cloned and bone marrow cells from susceptible mice are transfected with this gene to determine whether it can confer resistance to F-MuLV-induced early erythroleukemia.

Significance to Biomedical Research

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

Proposed Course of Research:

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

Previous studies have indicated that all strains of SFFV contain three major changes from their ecotropic parent, F-MuLV: an MCF gp70-related 5' end, a large deletion spanning the gp70-/p15E cleavage site, and a smaller and unique 3' end. Studies will be undertaken to assess the importance of each of these changes in pathogenicity.

(2) Further studies using the SFFV env gene vector.

Our studies have clearly indicated that the SFFV env gene in the absence of any other SFFV genes can, when introduced into erythroid cells, cause erythroleukemia. To rule out any involvement of helper virus proteins, the SFFV vector DNA will be prepared in the absence of helper virus, as previously done with the entire SFFV genome, and tested both in vitro and in vivo. The SFFV env gene vector should also provide an excellent vehicle for carrying on further genetic studies aimed at understanding the role of specific amino acid sequences within the gene. Following in vitro mutagenesis of the SFFV envelope gene, the DNA can be re-inserted into the vector and tested for pathogenicity.

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

Our studies have clearly demonstrated that the EcoRI to the ClaI fragment from the 3' end of the $SFFV_P$ envelope gene is responsible for the altered Epo responsiveness of $SFFV_P$-infected cells. We are now further dissecting this region into two subregions using the FokI restriction site. This site divides the 3' end of the SFFV env gene into EcoRI-FokI and a FokI-ClaI fragments, the later of which contains 120 bp from the extreme 3' end of the env gene which codes for the hydrophobic portion of the protein where the majority of the sequence differences between $SFFV_P$ and $SFFV_A$ reside. Recombinant viruses that contain either the EcoRI-FokI or FokI-ClaI regions from $SFFV_P$ on an $SFFV_A$ background are being prepared. These constructs will enable us to determine the significance of the hydrophobic region of the envelope glycoprotein to the phenotype of the virus produced. Subsequently, site specific mutagenesis will be carried out to actually pinpoint the exact sequence(s) that are essential for the altered Epo responsiveness induced by $SFFV_P$.

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

Erythroid cells normally require erythropoietin for proliferation and differentiation, and infection with SFFV apparently alters this requirement. Since our molecular studies continue to indicate that the envelope protein of SFFV, gp52, is the crucial element for biological activity, further studies are being planned to understand how this protein modifies erythropoiesis. Our studies to date have shown no evidence that the viral envelope protein is related to erythropoietin and protein sequence comparisons of the SFFV gp52 with erythropoietin show no homology. Studies being planned include (1) studies to compare the number of Epo receptors on $SFFV_P$-and $SFFV_A$-infected cells with uninfected erythroid cells; (2) studies to examine proteins phosphorylated in the presence or absence of Epo in virus-infected versus normal cells, and (3) studies to to determine the level of Ca^{++} uptake in response to

Epo in virus-infected versus normal cells. In addition to studies with Epo, studies will also be carried out to determine if the SFFV envelope protein is related to or acts in the same pathway as other growth factors or receptors involved in erythroid cell proliferation and differentiation. Finally, studies will be attempted to determine if SFFV is enabling erythroid cells to bypass the need for hormone/ receptor interaction by directly providing or inducing the second signal for proliferation and differentiation.

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

(5) Furthur studies on the multiple stages of SFFV-induced disease

The helper-free induction of SFFV-induced erythroleukemia provides an excellent model system for multistep malignant transformation. An advantage of this system is that progression of cells through stages of transformation is uncomplicated by the presence of infectious retroviruses which can continuously initiate new transforming events. Further experiments will look at mechanisms by which transformed cells that are not tumorigenic are transformed to a more autonomous, highly malignant phenotype. Initial studies will look at retroviral integration sites as well as rearrangements and increased expression of known onc genes. In addition, experiments are being planned whereby various onc genes can be transferred to cells in vitro or directly in vivo to determine if they are capable of driving cells to a more malignant phenotype.

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

Our previous studies with congenic mice have established that DBA/2, but not BALB/c, mice carry a gene on chromosome 5 at or near the Rmcf locus that plays a major role in resistance to early F-MuLV-induced erythroleukemia, and that expression of this gene is associated with the expression of an MCF virus-related envelope glycoportein on the cell surface that appears to block the receptor for MCF viruses. Further studies will focus on cloning the $Rmcf^r$ gene in order to determine whether it represents a structural gene for this unique envelope protein and to determine whether introduction of this gene into cells from susceptible mice will prevent the replication of MCF viruses and the development of F-MuLV-induced early erythroleukemia. While the resistance gene is being cloned, attempts will also be made to express a nonpathogenic FrMCF/ amphotropic virus envelope gene, which has been shown to bind to the FrMCF virus receptor, in a retrovirus vector and use this to confer resistance to susceptible strains of mice. Finally, an effort will be made to determine if this mechanism for resistance to retrovirus-induced leukemia in the mouse has relevance for retrovirus-induced diseases in humans. Studies have been initiated in collaboration with Dr. David Trauber, Downstate Medical Center, Brooklyn, New York, to prepare retroviral vectors containing the env gene of HTLV-III/LAV and to determine if such a vector will confer resistance to infection of human T cells with HTLV-III/LAV.

Publications:

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LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1985 through September 30, 1986

The activities of the Laboratory of Mathematical Biology (LTB) fall into several broad areas: macromolecular structure and function, membrane structure and function, immunology, pharmacokinetics, and computational and modeling methodology. The work is both theoretical and experimental. Application of theoretical understanding to these biological systems, which serve as models for aspects of the cancer process, is accomplished through the use of advanced computing. Close collaboration provides valuable feedback and knowledge transfer between these two research domains. The Laboratory consequently develops computer methodology that is utilized by researchers of the entire biomedical community.

Application of Sequences Analysis to Structure of Virus and Cell Proteins. In the Office of the Chief computerized analyses are used extensively along with techniques of biochemistry, virology, and electron microscopy to study picornaviruses, adenoviruses and other virus-cell systems. Sequences of picornaviruses, typified by polio, rhino, hepatitis, and foot-and-mouth disease, are examined for relationships within the family and to other known and hypothetical proteins. Secondary structures of the RNAs have been found to vary in correlation with pathological and sequence differences. Successful prediction of structure from sequence is confirmed by electron microscopic observation. Adenoviruses are studied with a goal to understanding early events in virus replication wherein the cell's metabolism is subverted to viral functions, and late events during which assembly and morphogenesis occurs. Early viral proteins, whose existence is known from biochemical studies, are analyzed by comparing their sequences to cellular proteins of known function. Physical association between the viral E3 glycoprotein and cellular MHC proteins correlates with homology between their sequences. Reovirus protein sigma-1 was found to have a heptapeptide pattern typical of a coiled-coil, alpha-helical structure (D. Chatterjee, J.V. Maizel, and J. Owens).

New analyses of proteins and nucleic acids are developed and implemented. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Methods to assess the significance of predictions use Monte Carlo simulations, evolutionary comparisons and biochemical data. Protein secondary structure is predicted from amino acid sequences. New sequences are compared with computerized databases to detect relationships with known proteins. A Government owned, contractor operated supercomputer laboratory has been established at the Frederick Cancer Research Facility under the sponsorship of this Laboratory. Equipment consisting of a Cray XMP supercomputer, VAX 8600 and 785 front end and graphic workstations became fully operational on April 1, 1986. Programs developed locally and

elsewhere for sequence analysis, structure prediction, chemical simulation and other procedures effectively utilize the combined capacity of the computers for a variety of molecular biomedical problems. This machine is the first scientific computer dedicated entirely to biomedical research (R. Nussinov, J.V. Maizel and J. Owens).

The computational scheme of DeLisi and Berzofsky (1985) used to associate α-helical amphipathicity with known immunodominant helper T-cell antigenic sites in proteins has been modified to produce algorithms for predicting T-cell antigenic sites (J. L. Cornette, H. Margalit, J. L. Spouge). The input data to an algorithm is the primary amino acid sequence of a protein. The algorithm identifies segments of the protein that would have a high probability of being an immunodominant T-cell site in the protein. The main characteristic of a segment that distinguishes it as a candidate T-cell site is that, if isolated as a polypeptide, it could form an amphipathic α-helix, but other correlates with immunodominant T-cell sites have been identified (presence of a lysine at the COOH-terminus and absence of any region with a propensity to coil conformation) and will be incorporated into future algorithms. To detect segments that have a propensity to form an amphipathic α-helix, each amino acid in the protein is assigned a hydrophobicity value (according to some scale) and the resulting sequence of numbers is searched for segments within which the numbers vary with a period of 3.6 residues per cycle. Both the hydrophobicity scale used to assign hydrophobicity values and the computational scheme used to detect 3.6 residue per cycle periodicity intrinsically affect the success of an algorithm. Thirty-eight hydrophobicity scale and two computational schemes — the discrete Fourier transform and a least squares fit of a sinusoid to the data — have been examined for their effectiveness in the algorithms. The algorithms are tested on a data base of 23 known T-cell antigenic sites in 12 proteins. The best algorithm uses the Fauchere-Pliska hydrophobicity scale and the method of least squares, and correctly predicts 18 of the 23 known T-cell sites with a probability of doing so by chance alone equal to .0006. The algorithms have been used to predict likely sites for T cell recognition of AIDS virus antigens and malaria parasite antigens, and some of the first peptides predicted this way are being made and tested.

Theoretical Molecular Structure. In the laboratory we are studying biological macromolecules and their properties. Both long range and short range interactions in proteins have been investigated by tabulating data from X-ray crystal structures. These studies have included solvent interactions. On this basis, we have derived hydrophobicity scales and effective interaction energies between all types of pairs of amino acids. We are applying these energies to locate hydrophobic nuclei and attempting to incorporate them into a protein folding scheme. Molecular modeling has been proceeding in four areas: membrane channels, DNA local conformations, DNA-carcinogen interactions and DNA-protein interactions. For the membrane proteins this model construction proceeds by combining experimental data with calculations of preferred locations and orientations of helices with respect to membrane boundaries, helix-helix packing, formation of charge pairs and disulfide bonds (H.R. Guy). Proteins that have been modelled are acetylcholine receptor, interleukin 2, δ hemolysin, α toxin and sodium action potential channel. Local sequence dependent DNA conformations have been proposed on the basis of energy calculations and solvent effects. (A.

Sarai, B. Shapiro, R. Nussinov and R. Jernigan). These results include both smooth and sharp bends. The effects of drug and carcinogens on the electronic structure of DNA are calculated (G. Barnett). For DNA-protein interactions, we evaluate the strengths of various types of DNA-protein and place the repressor in the most favorable orientation within the major groove of the operator region. Subsequent comparisons are made with experimental binding constants of various operators and their mutants; we investigate the specificties of the bindings to the operator, compared to the entire lambda DNA (A. Sarai Y. Takeda and R. Jernigan). This combination of modelling with experimental data has permitted a first derivation of detailed interatonic interaction energies from experimental binding constants. Further detailed evaluations are expected. Computer color graphics systems assist in these modeling efforts (K. Ting, R. Jernigan).

Simulation, Analysis and Modelling of Physiological Systems. The goal of this group (L. Zech, P. Greif and W. Fisher) is to understand the process of metabolites interacting with metabolic system by proposing and testing such systems within the framework of computational biology. This is done by examination of the synergistic interaction between the scientific methodology and the engineering paradigm in two projects: (1) The development of mathematical and computational tools for the simulation and analysis of biokinetic data and the implementation of these tools within the framework of SAAM and CONSAM, and (2) the development of theoretical models for particular physiologic systems which will lead to an enhanced understanding of the kinetic and dynamic behavior of these systems.

SAAM/CONSAM development and application has resulted in: 1) Development of a library of benchmark problems for SAAM/CONSAM were extended to include examples which can be used to verify the implementation of newer integration methodology into the program. 2) The development of an implicit function approach to local identifiability and estimability has results which begin to lead towards a practical solution of the optimal sampling problem. 3) In vivo transport of free cholesterol and esterified cholesterol among the components of blood was investigated resulting in: 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. 4) The metabolism of apolipoprotein B (apoB) in humans has been quantified more precisely using an integrated model which was developed for the analysis of the isotope kinetics of apoB in very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). 5) A detailed quantitative description of the metabolism of the core lipids of chylomicrons was proposed and the present hypothesis is being tested with data obtained from rats injected intravenously with radioactive chylomicrons metabolized by groups of recipient rats in the starved state, or else fed a single meal that was either fat-free or containing 5% fat. 6) The hyphothesis was tested that a physiologically based two-compartment model can predict independent estimates of NE production, local metabolism, and spillover consistent with known physiology and in meeting these constraints provides quantitative information about norepinepherine kinetics and compartment size in accord with what is known anatomically about the noradrenergic system. 7) LDL ApoB turnover kinetics has been reexamined and a new multicompartmental model of LDL metabolism was developed and is being tested using data from one normal and three hyperapoB subjects; each receiving a simultaneous injection of ^{125}I-buoyant

(L-LDL) and LDL^{131}I-dense LDL (H-LDL). The model underscores the physiologic importance of cholesterol-ester exchanges in the production and metabolism of H-LDL and L-HDL. 8) Selenium kinetics is important in cancer treatment and prevention. At the present time we are testing a selenium model using the pilot study data for oral selenate dosing. The current model is complex with four dissimilar plasma components, representing possibly three or four different selenium compounds, or 25 compartments when all the tissues are represented. It appears to be the simplest model to fit the pilot study data. It is undergoing minor modifications for its use as a hypothesis to examine the main study data.

Membrane Structure and Function. The studies in the Membrane Structure and Function Section (R. Blumenthal, A. Walter, O. Eidelman and M. Ollivon) deal with the the insertion and organization of molecules (proteins, lipids) in membranes, interactions of membranes with toxins, viruses, channel proteins and polypeptides, and membrane fusion. Spectroscopic techniques (fluorescence, circular dichroism) are used to study lipid-protein interactions and membrane fusion, and conductance across planar black lipid membranes (BLMs) is measured to approach questions of membrane destabilization and channel formation.

Studies on membrane fusion focus on reconstitution of viral spike glycoproteins into lipid vesicles and on membrane fusion mediated by the resulting virosomes. A rational basis for the reconstitution process is being developed by following dissolution by detergent, and reassembly of phospholipid and protein using fluorescence energy transfer techniques. Factors that control fusion (e.g. pH, surface charge, lipid headgroup, vesicle size, protein conformation) are examined in order to elucidate the fusion mechanisms. A theoretical framework is being developed to model aggregation and fusion kinetics (D. Covell, P. Greif). Efforts are directed at constructing virosomes which fuse with cells in order to deliver materials to the target cell membrane or cytoplasm.

Studies on channel-formation with cytolysin isolated from large granular lymphocyte tumors and from cytotoxic T lymphocytes are being pursued with lipid vesicles and planar bilayers to examine the mechanism of its lytic action. Recently it has been predicted that Interleukin-2 (IL-2) might form channels in bilayers (H.R. Guy). Experiments using planar bilayers and phospholipid vesicles containing voltage-sensitive dyes show pH-dependent and voltage-dependent permeability changes induced by this molecule. This finding might have important implications for the biological action of IL-2. In order to elucidate the mechanism by which adenovirus gains access to the cytoplasm of the host cell, via receptor-mediated endocytosis, its interaction with lipid membranes has been studied. The results indicate a pH-dependent permeability change in the liposomes, which parallels the pH-dependence of the disruption of endocytic vesicles by adenovirus.

Theoretical Immunology The programs of the Theoretical Immunology Section (J.N. 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; (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 50% theoretical and 50% experimental.

One project within the Section demonstrated the feasibility of delivering monoclonal antibodies via lymphatic vessels for diagnosis ("immunolymphoscintigraphy") and possible treatment ("immunolymphotherapy") of lymph node metastases and lymphoma (D. Covell, O.D. Holton, C.D.V. Black, M.J. Talley, J. Barbet, R.R. Eger, J.N. Weinstein). The initial studies were done in animals, using antigenic targets on both normal and malignant cells. Using the SAAM program package, kinetic models (D.G. Covell, R.R. Eger) were then developed. The Section is currently collaborating with other groups at NIH on trials (10 total) of lymphatic delivery in melanoma, T-cell lymphoma, Hodgkin's disease, 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.

Three other programs within the Theoretical Immunology Section are as follows: (1) The "percolation" problem (J.N. Weinstein, D.G. Covell, J. Barbet): 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 studies using a program package for solution of the partial differential equations. The analysis takes into account specific binding, nonspecific binding, and metabolism. One surprising prediction is that low affinity antibodies will sometimes be more effective in therapy than higher affinity ones. The theoretical predictions are being tested experimentally. (2) Immunotoxins and alpha-emitter/antibody conjugates (C.D.V. Black and J. Barbet): 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: An algorithm (HAL) has been developed with which to analyze secondary structural characteristics of peptides and to assess their potential for antigenicity. The results are being applied to HTLVIII/LAV envelope protein.

Image Analysis Work in the Image Processing Section (L. Lipkin) continued under three main headings: (1) Studies on 2D Gel Analysis (P. Lemkin) (2) Nucleic Acid Secondary Structure (B.Shapiro) and (3) the necessary hardware and software support (M.Schultz).

Collaborative work with Dr. Peter Sonderegger of Zurich as continued and involves both analysis of large 2D gel data bases as well as the development and implementation of new algorithms which are concerned with the detection of such subtle changes as charge pairs, etc. Further exploration and expansion of the Leukemia Data base in collaboration with Dr. Eric Lester at the University of Tennessee continues.

Extensive work on the PSAIL translator program makes likely that our expectation of near 100% Automatic SAIL to C conversion will be attainable. It is further clear that expansion of GELLAB by use of SAIL constructs with subsequent translation makes for a powerful programming solution to some difficult problems. Further much of the work on PSAIL is concerned with assuring external portability.

Work on nucleic acid structures has involved a variety of collaborations and directions. Included among these are those involving global sequence homology (R. Nussinov-Tel Aviv U.), the study of nucleic acid molecular helical twist,torsion angle, etc., particularly in terms of the energetics involved (Jernigan, Nussanov, Sairai), and a return to the problem of the analysis of structure as revealed in electron micrographs, but now as related to predicted shapes and gel migration patterns. Finally a beginning has been made on the problem of developing a Nucleic Acid Expert System, which would be directed to both intelligent queries by researchers, and a non-ad hoc structuring of the relationships among the various software/hardware complexes available at the Frederick center.

Membrane Biology The Membrane Biology Section addresses problems related to the topology, structure and dynamics of plasma and intracellular membranes as well as of intercellular junctions. In addition, it investigates the compactness and cytochemistry of cytoplasmic and extracellular matrices. To this end, it continues the development and application of freeze-fracture cytochemistry methods discovered in our laboratory: 1) fracture-label, for the cytochemical characterization of biomembranes and matrices after freeze-fracture; 2) label-fracture, for high resolution labelling of cell surfaces and its relation to the distribution of integral membrane proteins; and 3) fracture-permeation, for the assessment of the compactness of glutaraldehyde fixed cytomatrices through the permeation of macromolecular tracers into freeze-fractured tissues. Conventional freeze-fracture techniques are used to investigate the structure and biogenesis of intercellular junctions. The Membrane Biology Section has programs of collaboration with research groups at the Dept. of Anatomy, Univ. of Montreal (Canada), the Institute of General Pathology of the Univ. of Rome, School of Medicine (Italy) and the Laboratory of Renal and Vascular Pathology of the Broussais Hospital (France). Nationally, it collaborates with scientists at other NIH laboratories, at the Univ. of Maryland at College Park, and The Univ. of North Carolina, School of Medicine at Chapel Hill. Among others, current projects involve: 1) with fracture-label: distribution and topology of lectin receptors in intracellular membranes of duodenal columnar cells, of gangliosides in the plasma membranes of human neutrophils, of T_3 nuclear binding sites in liver cells, and of lectin binding sites within the extracellular matrices of rat and human glomeruli; 2) with label-fracture; high resolution localization of surface ligands in human lymphocytes, boar sperm cells and nerve cells in culture; 3) with fracture-permeation: the compactness of extracellular matrices in rat and human kidney glomeruli, in human neutrophils and in cardic muscle mitochondria.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08300-14 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 charectars 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:		
Waldo R. Fisher, M.D.,Ph.D.	IPA	LTB, NCI
Peter C. Greif, M.D.,	Staff Fellow	LTB, NCI

COOPERATING UNITS *(if any)*: Dr. Ray Boston, Murdoch Univ.,Australia; Dr. Trevor Redgrave, Univ. Western Australia; Dr. Charles Schwartz, Medical Coll. of Virginia,Richmond, VA; Dr. Bruce Patterson, Univ. of Fla., Gainesville; Dr. Barbara Howard, Phoenix

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	0.0

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.)*

Continuing development of a computer system (SAAM) for the simulation, analysis, and modeling of bio-kinetic systems. Development of a library of benchmark problems were extended to include examples which can be used to verify the implementation of newer integration methodology into the program. Performance studies with SAAM and CONSAM on the MicroVAX-II indicates that the MicroVAX has about 80% of the performance of the VAX 11/780 for a single user and that system performance while running SAAM and CONSAM is highly dependent on disk space management, the neglect of which can result in a 15 fold degradation in performance. Further development of SAAM/CONSAM resulted in the development of version 29 which will handle 3 times the number of data points.

Starting with a uniform method for testing local identifiability we have shown that an implicit function approach provides a common basis for examining local identifiability and estimability. These results lead towards a practical solution of the optimal sampling problem. The local identifiability based on linearization around initial parameter estimates has led to the examination of the information contained in the a priori correlation matrix, which is the correlation generated in the identifiability stage from error free generated data. Estimability has been posed as a measure of the independence of locally identifiable parameters when examined as the a priori correlation coefficients.

In vivo transport of free cholesterol and esterified cholesterol among the components of blood was investigated resulting in: 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 umol/min respectively). 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.

Cooperating Units (Continued):

Research Station, Phoenix, AZ; Dr. H. Bryan Brewer, Dr. Richard E. Gregg, Dr. Carlo, Gabelli, Dr. Dubo Bojanovski, Dr. Molecular Diseases Branch, NIHLB; Dr. Earnst Schaefer, USDA Human Nutrition Center, Tufts Univ, Boston Dr. Y-Da Chen, & Dr. 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. Mark Weaver, & Dr. Ronald Crystal, NHLBI, Dr. Oscar Lenaris & Dr. Jeffery Halter, U of Michigan. Dr. Ba-Bie Teng & Allen Sniderman, Royal Victoria Hospital, Monteral, Quebec. Dr. Gilbert Thompson, Hammersmith Hospital, London, England Dr. Stephen Dreskin, & Warren Strober, NIAAID

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.

Objectives:

The development of a general purpose computer program for the simulation of compartmental models of bio-kinetic systems which may be used by investigators not sophisticated in mathematics or computer programs. This development was initiated in 1959 by the late Mones Berman, and the program continues to be expanded and revised as new features are added, and old features are re-examined.

Methods Employed:

The goal of this research discipline is to understand the organization of metabolic systems including their connectivity and control. The specific aim of this research effort is to understand the processes involved in the realization of complex physiologic systems through computational solution of the analogic system. Since a physiologic system under investigation is subject to constraints, the power of the analogy is judged by the ability to satisfy these constraints and is associated with some measure of distance between the model and the physiologic system. Any change of connectivity among the compartments of the analogic system and any change in value of the flow between compartments of the model which result in a decrease of this distance, results in an improved analogy and by this measure a better realization of the physiologic system under investigation. The goal to explain accurately what takes place during a realization has been heuristic, based on the following rationale: when one solves a problem using a technique which previously worked, that technique becomes a method. When faced with a similar problem the method becomes a strategy. Over a number of years one develops a personal and idiosyncratic collection of problem solving strategies. By making systematic observations of problem solving strategies it is possible to identify and characterize a heuristic strategy. The coding of some of these strategies has resulted in SAAM and CONSAM. Other complementary and supplementary strategies remain to be coded. Other yet unknown strategies remain to be discovered in the process of analogic system realization.

Major Findings:

(1) Parameter Identifiability & Estimability: The overall problem of determining the parameter values of a system from input-output data is often called the identification problem. The identifiability problem, on the other hand, is more circumscribed: given a model of the system and specific input-output experiments, we ask, if the data were error free, would the parameters of the model be uniquely determined? The identifiability problem is concerned with the theoretical existence of unique solutions and is strictly a mathematical and a priori problem. Because it refers to specific experiments on a specific model, it is properly referred to as model identifiability for the given experimental design and is not system identifiability.

Identifiability of linear systems, and its extension to nonlinear systems, is just one aspect of a larger problem, the inverse problem, which includes identifiability and identification. In general the inverse problem is just the problem of science: determine the structure and function of a system from experiments and study of its behavior. Because of its inclusion in the inverse problem, identifiability is more than just an interesting problem in theory. From the point of view that we are interested in testing a particular hypothesis, we are interested in determining which parameters of a particular model are identifiable, not if the model is identifiable for a given experiment. Furthermore, a set of parameters may be identifiable but the interactions may be such as to make numerical estimation of the values of individual parameters difficult. One would like to design an experiment to optimize the estimability of the pertinent identifiable parameters. Identifiability, estimability, and optimal sampling design are linked steps in parameter estimation. If an initial estimate is available (which is often the case in biological problems) for a possible experiment, the iterative approach is: (a) test local identifiability at the initial estimates, (b) examine estimability of initial estimates, and (c) determine the optimal sampling design at the initial estimates. This requires only local identifiability of particular parameters.

Our long term goal is to develop a practical, uniform method for testing local identifiability and generating an optimal sampling design for the locally identifiable parameters which can be incorporated into SAAM. Starting with this formulation we have shown that an implicit function approach provides a common basis for examining local identifiability and estimability. These results lead towards a practical solution of the optimal sampling problem. The local identifiability based on linearization around initial parameter estimates has led to the examination of the information contained in the a priori correlation matrix, which is the correlation generated in the identifiability stage from error free generated data. Estimability has been posed as a measure of the independence of locally identifiable parameters when examined as the a priori correlation coefficients.

(2) Computational Procedures: As a prelude to the extension of the heuristic hypothesis and the formulation of other hypotheses, we sought to isolate the extent (frequency), duration, nature (machine type and mode), and diversity (range of investigations) of applications. This was undertaken with a view towards identifying areas of development. Of the approximate 1000 investigators

in 154 institutions using SAAM and CONSAM who responded with information; applications could be broken down into the following areas, and percentages.

FIELD	%
Pharmacokinetics	25
Metabolic systems analysis	18
Lipoprotein kinetics	13
Glucose/Insulin investigations	10
Curve fitting	9
Reaction mechanisms	8
Mineral metabolism	6
Heavy metal metabolism	5
Dosimetry	3
Numerical Analysis	3

Based on 76 requests for copies of the software from institutions outside the NIH in 1985 the following computer systems were employed in the testing of our software.

System	% (76)
Dec	
VAX	73%
10	1%
20	2%
IBM	20%
CDC	2%
Prime	—
Univac	2%
DG	4%
Honeywell	4%

In the study of biokinetic systems investigators use transient responses to aid in their understanding of the interconnections and rates of transfer of metabolites among constituent components of the system. This of course necessitates the solving of differential equations and within SAAM we provide the investigator with a flexible array of accurate and efficient numerical integrators. SAAM offers a two tiered support base; on one plane we have the identification of explicit systems and automatic invocation of a self-regulated Newton-Raphson solution procedure, whilst on the other plane an appropriate integrator can be selected from a set of three single step procedures and two multi-step procedures. With a view to: (a) validating the implementation of those procedures (b) determining both their default accuracies and their susceptibility to accuracy enhancement through solution tuning (c) isolation of their preferred application environment and (d) establishing their respective reliabilities and processing speeds a software project was undertaken which included: (a) the identification of numerical integration attributes which warrant monitoring in the sense that they may provide some gauge as to system

features interfering with integration performance (b) the establishment of comprehensive sets of test problems and (c) the implementation of the monitoring procedure in the SAAM software. Three sets of test problems have been assembled: "TEST01" is a set of problems which have been assembled to examine the fidelity with which SAAM interprets and processes biokinetic problems in terms other than direct solutions to differential equations. "TEST05 is a second test set of 15 differential equations selected to establish the robustness and accuracy of an integrator for any intended application. Each of the test problems in "TEST05" has a characteristic name and an accompanying set of "exact" (to eight digits) solutions which facilitates the comparison of solutions obtained with the SAAM simulator. In addition to "TEST01" and "TEST05", a third set of biokinetic models was chosen from the literature. Whereas the problems in "TEST01" and TEST05" call for 10-15 solutions per problem requiring 3 seconds of CPU, LIBRARY problems typically call for 150 solutions and run for 100 to 5000 seconds of CPU time. This test set reflects how well software performs in practical, model development, situations.

A small sample of performance results are shown in the three tables below.

Computers

#	Machine	Operating System	Fortran	Optimization
1	VAX 11/780 (4MB)	VMS 3.7	3.5	Yes
2	IBM 4341-2 (4MB)	VM/SP 3.0	VS F66	Yes
3	MicroVAX I (2MB)	Micro VMS 1.0	4.0	Yes
4	IBM 4341-2 (4MB)	VM/SP 3.0	VS 1.4 F77	No
5	MicroVAX II (2MB)	Micro VMS 1.1	4.2	No
6	IBM 4341-2 (4MB)	VM/HPO 3.2	VS 1.4 F77	No

Relative Speed Comparisons of Computers

		1	2	3	4	5	6
Chu-Berman	TEST01	3.11	2.17	13.96	2.35	4.45	1.00
	(SD)	(0.78)	(0.17)	(3.97)	(0.29)	(1.28)	—
	TEST05	2.78	2.24	8.16	2.36	3.70	1.00
		(0.19)	(0.13)	(1.50)	(0.11)	(0.58)	—
	LIBRARY	3.34	2.48	7.10	2.31	4.26	1.00
		(0.52)	(0.52)	(1.19)	(0.14)	(0.46)	—
	ALL	3.09	2.31	9.38	2.33	4.14	1.00
		(0.58)	(0.37)	(3.75)	(0.18)	(0.85)	—
Gear Procedure	TEST05	2.75	2.23	8.87	2.36	3.76	1.00
		(0.22)	(0.06)	(0.84)	(0.10)	(0.38)	—
	LIBRARY	2.69	2.12	7.24	2.16	4.01	1.00
		(0.25)	(0.12)	(0.86)	(0.05)	(0.13)	—
	ALL	2.72	2.18	8.09	2.26	3.88	1.00
		(0.52)	(0.52)	(1.19)	(0.14)	(0.46)	—

Relative Speed of Chu-Berman vs Gear Procedure Procedure

Computer #		1	2	3	4	5	6
	TEST01	4.40	4.52	3.51	4.53	-	-
	(SD)	(2.12)	(1.62)	(1.64)	(1.84)	-	-
	TEST05	4.29	4.25	4.82	4.33	4.31	4.44
		(1.53)	(1.27)	(1.79)	(1.44)	(1.38)	(1.48)
	LIBRARY	3.45	3.83	4.59	4.14	4.70	4.14
		(1.57)	(1.89)	(2.01)	(1.94)	(2.09)	(1.95)
	ALL	4.00	4.15	4.32	4.31	4.50	4.30
		(1.76)	(1.62)	(2.86)	(1.72)	(1.74)	(1.73)

The above tables present the first comparison between the Chu-Berman and Gear integrators for solving systems of differential equations. Clearly the Chu-Berman procedure is approximately four times faster than the Gear procedure for a wide range of numerical integration exercises and a wide range of systems of differential equations commonly characterizing the kinetic movement of

substrates and other entities monitored in biokinetic investigation. From this we infer that the kinetic systems describing such metabolic processes never become stiff enough to take advantage of the Gear procedure. This of course does not imply that such problems do not exist. For test sets "TEST01" and "LIBRARY" the local stiffness never exceeded 100. An examination of the accuracy of these integrators using "TEST05" has shown that the Chu-Berman procedure is more efficient than the Gear procedure.

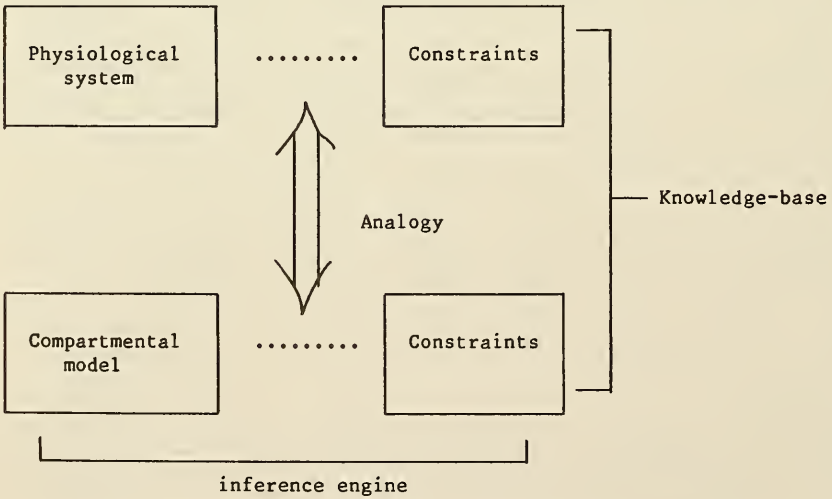
Processor evaluations have revealed surprisingly good performance for the new wave of computers becoming available. Relative to the mainframe 4381-2, the MicroVax I has 10%, and the MicroVAX II has 25% of the solution speed for the SAAM software. While the performance of microcomputers relative to mainframes declines as the number of concurrent users increases, for an isolated investigator modestly priced microcomputers clearly provide adequate hardware support for biokinetic investigation.

Associated with conversions of software to alternate computers, upgrading existing command structures, or inclusion of additional commands into the repertoire, the need to verify the integrity of the interactive commands has developed. Using an extended test problem and an accompanying command set of 130 variations of 90 plus CONSAM commands, the software can be exercised and the results tested against a verified response for identity. This tool ensures that the most common commands, in their most likely and most frequent environment, can be tested and that each functional module of the interactive environment was not only represented in a test but sequentially represented in an array of appropriate environments.

Software developments can be divided into interactive development (code enhancements which render the software easier to use) and model processing development (code enhancements which extend the scope of the software application). When we view an analogic model as a number of structures including parameters, equations, state variables, observed data, etc, SAAM/CONSAM renders the structures available as a data base to examine and modify as the situation demands. The extent of this data base reflects the size of the load module while the partitions of the database among the structures reflects its allocation of support to the activities defined by the structures. In the past, because of the closed nature of the SAAM modules neither the database extent nor the infrastructure partition was adjustable. While the original structure with its associated rigidity worked well for the investigation of typical models using modest size computers, it was not sufficiently extensive for larger models and associated observation sets nor sufficiently flexible to be tailored for use on minicomputers. Because of the complicated inter-relationships among the dependent structural extents and because of the large number of structures in the SAAM database, the reconfiguring of the database is more than reassignment of parameter values. We have designed a stand-alone database configurator with the following characteristics: (a) interactive selection of all SAAM/CONSAM independent data structure extents, (b) determination of the dependent structure extents through logical operations on the independent data structure extents, (c) updating of the "include" files in the SAAM/CONSAM source code, (d) compilation and linking of the object modules, and test the new image using the test files "TEST01","TEST05", and "LIBRARY".

Proposed Course:

Future Plans: The heuristic approach while successful can only be improved by moving to a new heuristic, improving the tools to carry out the heuristic, or moving to a new environment which increases the execution speed of the heuristic. Over the next three to five years, we propose to conduct an examination of the techniques of compartmental model construction and testing from the point of view of cognitive psychology and artificial intelligence (AI). While the concepts are of interest in many diverse fields from philosophy to linguistics, Bwe intend to focus these concepts on the task of physiologic system realization through the analogy of a compartmental model. Knowledge-based systems are two dimensional in that they are made up of two central components, the knowledge-base and the inference engine. A variety of AI techniques have been used to construct knowledge bases including rules, frames, nets, predicate calculus, and procedures. Depending on the physiologic system under investigation, most of these can be used; however, compartmental models may be more restrictive than the general case. The inference engine is applied in parallel to the model system and its constraints. We plan to examined the sharp distinction between the knowledge-base and inference engine with two clear consequences. First, it makes possible the use of different knowledge bases. This means that the methods are applicable to more than one physiologic system. Second, this distinction facilitates the expression of what should be done with the inference engine, with less attention to how it should be done. A major thrust of this research effort is to provide the ability to build approaches which allow the possibility to reason from the fundamental mechanisms of physiology, biophysics, and biochemistry. It has been stated "the key issue is understanding, modeling, and reasoning about causality: a central concept in many (if not all) of the (research) efforts (on inference engines) is the notion of causal explanation".



Project Description #2:

Project #2 Application of SAAM and CONSAM to the Simulation and Analysis of Bio-kinetic Data.

Objectives:

To develop general purpose mathematical models for particular physiologic system which will lead to the understanding of the kinetic-dynamic behavior of the systems. After the models are developed they are then used to separate out specific parameters of the systems and through drug and diet modulation of the physiologic system obtain a relationship between the kinetic parameters and the dynamic behavior of the system under study. While this is not always the case, many of the models are developed to further understand the response of a physiologic system to an injection of a tracer substance. These objectives have been met through the study of the following systems listed in this report and others for the Laboratory of Mathematical Biology.

Further the analysis of data from particular physiologic systems provides a window through which the ongoing need of the users of SAAM and CONSAM can be assessed. This assessment is then combined with mathematical techniques to become the starting point for further development in project #1 above.

Methods Employed:

When turnover data first becomes available, preliminary analysis consists of fitting the plasma decay curve with a mathematical function, usually a sum exponentials, and determining the area under the decay curve. After extrapolation of the curve back to zero time, the volume of distribution for the isotopic tracer is calculated from dilution and checked for consistency against an independent estimate of the patient's volume. If the difference between the estimate of volume of distribution is small, the area under the decay curve is used to calculate residence times for the before constructing a compartmental model. If, however, the difference is large, calculations of the residence time is not completed until sufficient data has been collected and a compartmental model has been constructed.

The methods used for the development of multicompartmental models using turnover data from tracers and pharmaceuticals have been detailed in project #1 above. These models are simulated using the SAAM simulator (a large collection of digital computer programs run on the Digital VAX-11/780 computer in the Laboratory of Mathematical Biology, National Cancer Institute. These simulated results are compared to the experimental results and the connectivity (number and topology of compartments) as well as the flow of tracer or tracee in the model changed until a working model is developed. Using the model, the volume of distribution of the tracer or drug is estimated and compared to independent estimates of these volumes. After development of the compartmental model, the parameters of the model are adjusted using nonlinear least squares techniques resulting in minimal least square error. These have now been extended to studies in which differences in multicompartmental models, developed using turnover data from two separate radiolabels, or by two different routes of administration in normal and treated individuals, have been compared.

Major Findings:

(1) In collaboration with Dr. Beltz, Dr. Howard, & Dr. Grundy we have begun to quantify more precisely the metabolism of apolipoprotein B (apoB) in humans, using an integrated model which was developed for the analysis of the isotope kinetics of apoB in very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). The experimental basis for model development was a series of 30 triple-isotope studies in which patients received autologous ^{131}I-VLDL, ^{125}I-LDL and [^{3}H]-glycerol as a precursor of VLDL triglycerides. The currently proposed model contains the following components: (a) a VLDL delipidation cascade that has a variable number of subcompartments, (b) a slowly catabolized pool of VLDL, (c) and IDL compartment consisting of two closely connected subcompartments, one of which is outside the immediate circulation, and (d) a two-compartment subsystem for LDL. Because mass data indicate that not all VLDL were converted to LDL, the model allows for irreversible removal of apoB from VLDL (or IDL) subsystems. The model is consistent with specific activity curves from the triple-isotope studies and with present concepts of lipoprotein physiology. The model has been used to quantify pathways of lipoprotein apoB transport in normal and abnormal states.

(2) In collaboration with Dr. Redgrave a detailed quantitative description of the metabolism of the core lipids of chylomicrons was developed with data obtained from rats injected intravenously with radioactive chylomicrons. Groups of recipient rats were starved or else fed a single meal that was either fat-free or containing 5% fat. The kinetic model included chains of delipidation compartments, and a remnant particle. Compartments for recirculation of portions of triglyceride, cholesteryl ester, and unesterified fatty acid radioactivities were all included and necessary to obtain good fits to the data. Making the simplifying assumption that remnants could be represented as particles retaining cholesteryl esters but depleted in triglycerides, a 'delipidation index' was calculated as the ratio of the triglyceride residence time divided by the cholesteryl ester residence time. Significant differences were found in the delipidation index between the starved group and both the normal-fed and fat-free fed groups. Compared with rats fed a meal of the usual diet which contained 5% fat, starved rats showed more extensive delipidation, but a longer residence time of remnant particles. A longer residence time of triglycerides in rats fed a fat-free meal was explained by less delipidation, but remnant removal was accelerated. The kinetic model makes possible an interpretation of the patterns of metabolism in terms of the known underlying physiological mechanisms.

(3) 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.

(4) In collaboration with Dr. Linares and Dr. Halter we tested the hyphothesis that human norepinephrine (NE) kinetics can be described by a physiologically reasonable two compartmental model. Steady-state NE specific activity in arterialized plasma during 50 min. ^{3}H-NE infusion (15μCi/m^2 bolus plus 0.35μCi/m^2/min) and 50 min. postinfussion plasma disappearance was measured in eight healthy subjects in both supine and during upright posture. Two exponential components were clearly identified in the plasma ^{3}H-NE disappearance curves of each subject studied both in the supine (r values 0.94 to 1.00, all p<).01) and upright (r values 0.90 to 0.98 all p>0.01) positions. A single exponential term did not fit the data well (r values 0.68 to 0.75, all p>.1) as previously reported by many investigators for several decades. Partial F-test analysis of the exponential curve fits for each subject in the supine position and the upright posture revealed the biexponential fit to be superior in explaining the data (all p>0.01).

Simultaneous computer analysis of all the experimental data for each subject established that a minimal-change two compartmental model is the minimal model necessary to account for the kinetics of NE both in the supine position and during upright posture. The rate of production of NE into compartment 2 (the extravascular compartment) estimated using the two-compartment model increased with upright posture (1.87±.08 vs 3.25±.2 μ/min/m^2, p>0.001). The estimated percent of NE metabolized in compartment was 85±1% with only 15±.6% spilling over into compartment 1 (plasma-containing compartment). The estimated mass of NE in compartment 1 was much lower than in compartment 2 both in the supine (0.62±.05 vs 82±3 μg/m^2 p<0.001) and upright (1.01±.08 vs 125±29 μg/m^2 p<0.001)positions. With upright posture there was a modest increase in NE mass in compartment 1, but a very large increase in NE mass in the extravascular compartment (p<0.001). Upright posture was associated with a fall in the volume of distribution of NE in compartment 1 (7.5±.6 vs 4.7±.11 liters, p<0.001). As a result, the metabolic clearance rate of NE from compartment 1 decreased with upright posture (1.80±.11 vs 1.21±.08 L/min/m^2, p<0.001).

We conclude that a physiologically based two-compartment model can predict independent estimates of NE production, local metabolism, and spillover consistent with known physiology and in meeting these constraints provides quantitative information about norepinepherine kinetics and compartment size in accord with what is known anatomically about the noradrenergic system. Physiologically based

compartmental analysis of plasma NE kinetics provides a new radioisotopic approach for the study of NE metabolism in normal physiology and disease states in humans.

Also 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 extravascuar 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.

(5) In collaboration with Dr. Y-Da Chen, & Dr. Gerald Reaven; The effect of alloxan-induced insulin deficiency on high density lipoprotein metabolism was studied in rabbits. Rabbits with alloxan-induced diabetes mellitus had significantly higher (mean±sem) plasma concentrations of glucose (541±13 vs 130±2 mg/dl, p<0.001),triglyceride (2851±332 vs 101±10 mg/dl, p<0.001),and total plasma cholesterol (228±55 vs 42±4 mg/dl, p<0.001) than did normal control rabbits. However plasma HDL-cholesterol concentration was lower (7.2±1.0 vs 15.3±1.3 mg/dl, p<0.001) in the diabetic rabbits. HDL kinetics were compared in diabetic and control rabbits, using either ^{125}I-HDL or ^{125}I-AI/HDL, and demonstrated that HDL fractional catabolic rate was slower and residence times longer in the diabetic rabbits when either tracer was used. Thus the reduced plasma HDL-cholesterol concentrations seen in rabbits with alloxan-induced insulin deficiency was associated with a lower total sysnthesis rate. Since insulin treatment restored to normal all of the changes in plasma lipoprotein concentration and kinetics observed in diabetic rabbits, it is unlikely that the phenomena observed were secondary to a non-specific toxic effect of alloxan. These data strongly support the view that insulin plays an important role in regulation of lipid metabolism as well as glucose metabolism.

(6) In collaboration with Dr. Ba-Bie Ting, Dr. Gilbert Thompson, Dr. Peter Kwiterovich, & Dr. Allen Sniderman LDL ApoB turnover kinetics has been reexamined. 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 recieved 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.

(7) 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 are 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 ehich 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.

Soon we will begin applying this model to the main study subjects. Then we will begin following similar procedures to develop a model for selenomethionine. 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.

Proposed Course:

Detailed studies will be continued on the analysis of the differences in apoA-I and apoA-II metabolism by further specifying and defining the current compartmental models, with particular emphasis on analysis of studies in abnormal subjects such as type I and Tangier subjects. In addition analysis of data form studies using endogenous labeling (tritiated leucine) will be used to test present hypothesis regarding apo A-I metabolism. The overall objective will be the development of a comprehensive model of human lipoprotein metabolism. The formulation of an overall conceptualization of lipoprotein metabolism will be continued by qualitative and quantitative testing of these conceptions using compartmental model, statistical and other theoretical methods. Of particular interest will also be the

determination of which parameters are modified by diet, drug and transformed by genetic disease.

Compartmental models for selenium metabolism will continue to be developed so that differences in the absorption and metabolism of the inorganic and organic forms can be compared at a machinistic level. These models will be further developed and used to investigate effects of fasting on absorption and metabolism of both the organic and inorganic supplemental dietary selenium.

Compartmental models for the metabolism of neurotransmitters will be extended to include results from recient kinetic studies involving epinephern by its self and in combination with norepinephern.

Significance to Biomedical Research and the Program of the Institute:

One of the major misunderstandings of the biological sciences revolves arround the fact that a few grams of neoplastic tissue can take over the entire homeostatic control of a 70kg man and lead to his death. Understanding this process is the two pronged approach of understanding the basic physiology and control mechanism and understanding how the neoplasm modulates these basic mechanisms. Using this understanding then the institute will be in a position to design and test cancer prevention and cancer treatment strategies.

Understanding the mechanisms of metabolism of tracees and pharmacuticles and the moieties which up these are significant because of their relationship to physiology and pathology of honeostasis and disease. The development of a concept of metabolism is approached by the theoretical analysis of data from metabolic studies using the techniques of compartmental and statistical model building. This type of theoretical analysis provides a framework for discussion between investigators. This project consists of the testing and further development of compartmental models for metabolism and pharmacokinetic as well as the proposal of new models where they do not exist.

Understanding the modulation of lipoprotein, cholesterol, cholesterol ester, triglycerides and apolipoproteins by drugs, diet and genetic disease is also of significance since changes in these effectors may have major effects on atherosclerosis and cholelithiasis as well as malnutrition in cancer and other neoplastic disease. This theoretical analysis also provides a framework for comparison between groups as dissimilar as caucasians and American Indians.

In addition the metabolism of lipids, particulary triglyceride and FFA's, is associated with cancer risk as well as nutrition in the subject with cancer. Tumor necrosis factor is known to bind to fat cells and reduce the secretion of lipoprotein lipase which in tern modulates triglyceride and FFA metabolism.

Furthermore, it is intended that the study of the lipoprotein metabolic system serve as a particular system in which to develop the techniques of analysis to large (more than 20 simultaneous differential equations) metabolic systems these findings can later be applied to many different systems.

Understanding metabolism of selenium is of major importance due to the central role of in normal and patients with neoplastic disorders and their prevention Because of the recent elucidation of the positive correlations between selenium levels and risk of developing cancer the understanding of the absorption and metabolism of the two major forms of selenium are particularly relevant to the understanding of cancer.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08303-14 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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

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

Robert Blumenthal, Ph.D., Chief, Membrane Structure & Function Section, LTB, NCI

Other Professional Personnel:

Anne Walter, Ph.D., Staff Fellow	LTB, NCI
Ofer Eidelman, Ph.D., Visiting Fellow	LTB, NCI
Anu Bali, Ph.D., Visiting Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Pierre Henkart, IB, NCI; Dr Ira Pastan, LMB, NCI; Dr. Steven J. Morris, IRP, NINCDS; Dr Manfred Schubert, NINCDS; Dr Joseph Handler, NHBLI. Dr Michel Ollivon, CNRS, France, Dr Abraham Loyter, Hebrew University, Israel.

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Membrane Structure & Function 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.)*

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) and the HN and F proteins of Sendai 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) analysis of factors which determine approach of virus to target (electrostatics, steric constraints); iv) analysis of factors which promote membrane deformation and destabilization (liposome size, lipid composition, osmotic forces); v) Examination of possible effects of pH-gradient or membrane potential on fusion rates; vi) analysis of the role of cooperativity of viral glycoproteins in mediating fusion; vii) Examination of the disposition of the fusion protein after the fusion event; viii) Identification of possible fusion intermediates; ix) Development of methods to study fusion activity of mutants of viral proteins using cloned viral membrane protein sequences expressed in transfected cells; x) Structural studies of viral proteins; xi) Development of methods for using reconstituted viral envelopes as vehicles for specific delivery of materials into cells.

Project Description:

Major Findings:

1) In order to study kinetics and extent of fusion of intact virions with liposomal and cellular targets, we have labeled Sendai virus with octadecylrhodamine B chloride. Fusion is monitored by fluorescence dequenching as the probe spreads into the target membrane. Inhibition by phenylmethylsulfonyl fluoride and dithiothreitol, which are known to block viral fusion with biological membranes served as tests for the validity of the assay. We showed inhibitable fluorescence dequenching with red cell ghosts, hepatoma tissue culture cells and liposomes made of PC/cholesterol as targets.

2) We have studied pH-dependent fluorescence dequenching of octadecylrhodamine-labeled VSV interacting with monkey kidney (Vero) tissue culture cells. The pH-dependence of fusion matched that of VSV-mediated cell-cell fusion. Fusion was blocked by neutralizing antibody. At neutral pH we observed fluorescence dequenching, which occured at a slower rate than the dequenching at acidic pH. This signal represents fusion of the virion in an acidic endosome, since it was blocked by agents such as NH^4Cl and methylamine.

3) We have studied pH-depend fusion of intact VSV with fluorescently labeled liposomes containing negative charge (cardiolipin or phosphatidylserine). There was a transition in the extent of fusion at a pH of about 6. This matches the pH-dependence of VSV-mediated cell-cell fusion.

4) In order to develop a rational basis for reconstitution of viral spike glycoproteins, we have continued our studies on the definition of the phase diagram of the phosphatidylcholine-octylglucoside-VSV G system. We find that in the presence of lipids or proteins the apparent critical micelle concentration (cmc) is lowered, and, in fact one is dealing with a critical mixed micelle concentration (cmmc). From our data we propose three domains for the egg-PC/octylglucoside system: a) L(D), a phospholipid lamellar phase with dissolved detergent ; b) M(L), a detergent micellar phase with dissolved phospholipids ; and c) $L(D) \rightleftarrows M(L)$, a domain of equilibrium between two populations: lamellar phase saturated with detergent and micellar phase saturated with lipid. At high lipid concentrations we noted the appearance of phase separation in the mixture. Bilayer reconstitution in the presence of VSV G-protein exhibited a similar phase diagram to that seen in the absence of protein.

5) We developed a methodology to monitor surface charge using Co^{2+} as a collisional quencher of fluorescent lipid probes incorporated into liposomes. Using the Gouy-Chapman-Stern equation we measured the surface density of Co^{2+} bound to negatively-charged lipid. In addition, the ability of Co^{2+} to penetrate the flourecsently labeled liposomes was monitored as a measure of stability of those liposomes.

6) We have applied aqueous content mixing assays of ion-induced fusion to rapid kinetics using stopped flow mixing. One such assay involves a fluorescence increase as a result of formation of the chelation complex between terbium and dipicolinic acid which occurs when vesicles loaded with terbium fuse with vesicles loaded with dipicolinic acid; the other (more appropriate for studies at low pH) utilizes quenching of aminonaphtalene-3,6,8-trisulfonic acid by N,N -p-xylylenebis (pyridinium bromide) encapsulated in two separate populations of vesicles. We compared our stopped flow data with the conventional handmixing experiments, and concluded that the initial 5 seconds can not be resolved by the latter method. These results will have important implications for our kinetic studies of viral fusion.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08320-11 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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

COOPERATING UNITS *(if any)*: Dr. J. Ferretti, Laboratory of Chemistry, NIHLB; Dr. F. Wang, National Bureau of Standards, Gaithersburg, 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:
0.1	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.)*

Statistically derived Phi-psi maps for each type of residue indicate substantial improvements in X-ray data over previous tabulations.

Position effects in regular secondary regions show strong effects for some types of residues, especially proline, aromatic and polar groups.

A simple dipolar solvent model indicates an asymmetry to electrostatic interactions. Favorable interactions appear to be enhanced and extended to longer range.

NMR investigations of an ACTH fragment indicated no dominant conformations. As with most small peptides, it was highly flexible.

Project Description

Objectives:

The aim is to study the conformations available to short peptides and, in a systematic way, develop better secondary prediction methods. Studies of homologous proteins can indicate permissible substitutions of amino acids.

Methods Employed:

The conformational properties of peptides are not sufficiently understood. In some cases, single substitutions of amino acids can frequently cause significant changes in conformation beyond those expected from existing theories; whereas in other cases similar substitutions probably cause no significant change. Based in part on our previous studies of protein secondary structures, we have been pursuing studies on the sequence dependence of conformation in several ways. These have included statistical surveys of X-ray crystal data to investigate the position dependences of amino acids in secondary structures, tabulation of statistical Phi-Psi maps, simulation of solvent with a dipole model in the vicinity of charge pairs, and selection of peptides for study by experiment and calculation. In the latter case, measurements of intramolecular distances by 2D NMR and fluorescence energy transfer measurements are planned. These would serve as constraints in calculations of accessible molecular conformations.

Major Findings:

The additional crystal structures available since previous published tabulations of statistical Phi-Psi maps permit substantial improvements in the appearances of these maps. This is manifested in several ways: the maps are substantially smoother and also, there are clearer distinctions between the beta domains of the different residues. Unfortunately there is still not sufficient data to obtain useful maps for many specific pairs of amino acids.

The position dependent statistics of amino acids in secondary regions manifest a number of interesting features not frequently noted before. The most obvious features are the prominence of proline at the amino terminus of helices and the high probability of asparagine at the carbonyl end of beta strands. Numerous other weaker position effects also appear.

Simulations of the interaction energy of ion pairs with a model dipolar solvent indicate that at relatively close distances the energies of interaction of charges of the same and opposite signs are not simply equal in magnitude but opposite in sign as for simple Coulombic interactions. Instead, the opposite signed charges interact more favorably and to longer distances than expected because of the possibility of favorable arrangements of the intervening solvent dipoles.

Investigations of close hydrophobic pairs of amino acids have indicated that they diminish in number monotonically from the center to the exterior of globular proteins. Methods were developed to combine results from large numbers of diverse proteins.

2 dimensional NOE studies of the ACTH fragment 4-10 indicated no cross peaks, and hence no dominant interactions or dominant conformations other than those expected from the chemical bonds.

Significance to Biomedical Research and the Program of the Institute:

Development of a reliable method for predicting peptide conformations from their sequences would permit a better understanding of protein and peptide structure and function.

Proposed Course:

For some other short specific sequence peptides, we will obtain fluorescence intra-molecular distance measurements and 2D NMR distance measurements to combine with calculations of possible conformations. This combination of experimental distance constraints should serve to eliminate sufficient numbers of the large number of conformations and yield average properties of the preferred conformations. In addition we hope to obtain information about the temperature and solvent dependences of such preferences.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08335-10 LTB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

"Targeting" Liposomes for Selective Interaction with Specific Cells and Tissues

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

John N. Weinstein, M.D., Ph.D.	Senior Investigator	LTB, NCI
Other Professional Personnel:		
Oscar D. Holton, III, Ph.D.	Expert	LTB, NCI
Jacques Barbet, Ph.D.	Guest Researcher	LTB, NCI
Bobak Mozayeni	Howard Hughes Fellow	LTB, NCI
Christopher Black	Visiting Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

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	0.5	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 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 HTLVIII/LAV-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 selectively deliver MTX to mouse tumors in vivo and inhibit their growth.

(3) Compartmental targeting. We have demonstrated the delivery of liposomes and entrapped drug to lymph nodes after subcutaneous and intraperitoneal, injection and have determined cellular sites of localization. These studies have been extended to antibody-bearing liposomes.

Project Description

Objectives:

To investigate the use of liposomes both in cell biology and in clinical therapy. Within this broad context, to (1) explore the use of antigen-antibody interactions to achieve selective association of liposomes with particular cell types; (2) develop synergistic interactions between "temperature-sensitive" liposomal drug carriers and hyperthermic treatment; (3) identify the mechanisms of spontaneous, serum-induced and osmotically-induced release of solutes from liposomes; (4) deliver liposomes and their contents to tumor in lymph nodes — for nonspecific and antibody-mediated binding; (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 HTLVIII/LAV-infected cells.

Non-standard Methods Employed:

(a) Preparation of liposomes, by bath and probe sonication; (b) investigation of liposome-cell interactions using the fluorescence-activated cell sorter; (c) dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory for this purpose; (d) measurements of release of solute from liposomes by "fluorescence self-quenching"; (e) determination of the mobility of vesicles bound to cells, using fluorescence photobleaching (f) microwave heating of tumors; (g) quasi-elastic light scattering measurements of liposome size; (h) heterobifunctional covalent coupling of antibodies to liposomes.

Major Findings:

Objective 1: (a) Bivalent antibody selectively binds TNP-bearing liposomes to TNP-bearing lymphocytes, but the binding does not increase delivery of liposome contents to the cell interior (beyond the amount internalized spontaneously). (b) Endogenous surface IgA on cells of the murine myeloma MOPC 315 can bind liposomes bearing the appropriate hapten (DNP) to the cell surface. However, as in the lymphocyte system, binding does not increase delivery to the cytoplasm. (c) Lipid vesicles containing fluorescent molecules are potentially useful as markers for sparse or low-affinity cell surface determinants. They can be made to contain many fluorophore molecules, thus amplifying the signal. They give very low-nonspecific background since the fluorophore is sequestered, and appropriate lipids are not very "sticky". (d) Liposomes are removed from the circulation of a mouse much faster if the mouse carries a myeloma secreting antibody to a hapten on the liposomes. (e) Antibody-mediated binding of methotrexate-containing vesicles to MOPC 315 and TEPC 15 myeloma cells did not lead to entry of drug into the cells and inhibition of their metabolism. (f) IGG opsonized DNP-vesicles are bound in large numbers to F_c receptor-bearing cells (murine $P388D_1$), and are then endocytosed. Liposome-encapsulated MTX can escape the pagolysosomal system to reach a cytoplasmic target (dihydrofolate reductase) and affect the physiology of the cell. (g) IgG, protein A, avidin, and other ligands can be coupled efficiently to liposomes by use of the heterobifunctional

cross-linking agent N-hydroxysuccinimidyl 3-(2-pyridyldithio) propinate. This method of coupling results in only minimal aggregation and little leakage of vesicle contents. Liposomes bearing covalently coupled mouse monoclonal antibody against human α_2-microglobulin bind specifically to human cells, but not to mouse cells. (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 08367-02 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.

Significance to Biomedical Research and the Program of the Institute:

The three objectives listed relate to the possibilities of using liposomes in diagnosis and tumor therapy. A major barrier to such efforts has been the difficulty of directing liposomes to particular cells or anatomical sites. The studies of antibody-mediated "targeting" suggest a way to achieve selectivity but also demonstrate an additional problem: how to get the liposome and its contents into the cell after binding. The studies of synergism between liposomes and hyperthermia indicate a new way to achieve selective delivery. Use of the liposome as a hapten-carrier for analysis and sorting of cell populations may be useful in a number of areas of cellular and tumor immunology. The covalent attachment of immunoglobulin and ligands to liposomes will make possible a wide range of cell biological studies. Liposomes may be able to carry diagnostic and therapeutic agents to tumor in the lymph nodes.

Proposed Course:

This project has been phased-out in this fiscal year except for the publication of manuscripts and for new developments described under Objective 1, using monoclonal antibodies covalently bound to liposomes directed against HTLVIII/LAV-infected cells..

Publications:

Klausner, R.D., Blumenthal, R., Innerarity, T., and Weinstein, J.N.: The interaction of apolipoprotein A-I with unilamellar vesicles of L-α-dipalmitoyl phosphatidylcholine. J. Biol. Chem. 260: 13719-13727, 1985.

Weinstein, J.N., and Leserman, L.D.: Liposomes as drug carriers in cancer chemotherapy. In I.D. Goldman (Ed.): The International Encyclopedia of Pharmacology and Therapeutics. New York, Pergamon Press, in press.

Weinstein, J.N., Parker, R.J., Holton, O.D., III, Black, C.D.V., Sieber, S.M., and Covell, D.G.: The pharmacology of monoclonal antibodies. In R. Reisfeld (Ed.): Membrane-mediated Cytotoxicity, Alan R. Liss, Inc., in press.

Weinstein, J.N., Blumenthal, R., and Klausner, R.D.: Carboxyfluorescein leakage assay for lipoprotein-liposome interaction. Methods in Enzymol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08341-08 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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

Studies of Lipid-Protein and Protein-Protein Interactions of HTLVIII/LAV

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

John N. Weinstein, M.D., Ph.D.	Senior Investigator	LTB, NCI
Other Professional Personnel:		
Robert Blumenthal, Ph.D.	Chief, Membrane Structure & Function Section	LTB, NCI
Bobak Mozayeni	Howard Hughes Fellow	LTB, NCI
H. Robert Guy	Expert	LTB, NCI
Kai-Li Ting	Computer Programmer	LTB, NCI

COOPERATING UNITS (if any)

Dr. T. Innerarity and Dr. R. Pitas, University of California at San Francisco; Dr. Richard Klausner, LBM, NIAMDD; Dr. R. Schwartz, LI, NIAID; Dr. G. Shearer, IB

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Theoretical Immunology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.3	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.)

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 atheroscleroic plaques.

A statistical mechanical algorithm (HAL) was devised for evaluating amphipathic helical structures 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 HTLVIII/LAV. Lipid membrane systems and human cell isolates are being used experimentally to investigate the interaction between characterized synthetic antigenic peptides and T-cells in the recognition process. The results may have application to the design of vaccines.

Project Description:

Objectives:

To investigate the interaction between liposomes and lipoprotein and between lipoproteins and cells. More specifically, (1) To define the mechanism by which liposomes are broken down by serum components, principally the lipoproteins; (2) to determine the relative efficacies of different purified apolipoproteins in breaking up liposomes; (3) to develop fluorescently labelled lioporoteins and liposome-apoprotein recombinants for use in studying lipoprotein-cell interactions; (4) to use the methods developed for objectives (1) - (3) to assess the physical chemistry of protein-lipid interaction in lipoproteins; (5) to extend to other bilayer-protein interactions the concepts thus developed; (6) in particular, to investigate the assembly of tubulin and actin into membranes; (7) to study physical chemical aspects of the interaction of proteins with lipid bilayers; (8) to study physical chemical aspects of of T-cell antigenicity.

Non-standard Methods Employed:

(a) Preparation of liposomes, by probe and bath sonication: (b) Dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory, i.e., "phase transition release" (PTR); (c) Determination of the mobility of lipoproteins and liposomes bound to the cells, using fluorescence-photobleaching recovery; (d) Neutron scattering studies of lipoprotein size and shape; (e) Aqueous collisional fluorescence quenching studies of lipid-protein interactions; (f) Derivatization of proteins with trinitrobenzene sulfonate; (g) Labelling of lipoproteins with 3,3'-dioctadecylindocarbocyanine (diI) dye; (h) HAL (Helical Amphipathicity Locator) programs for analysis of peptide secondary structure.

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, diI. 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) Use of the HAL algorithm demonstrates that the correlation between helical amphipathicity and T-cell antigenicity is not strong.

Significance to Biomedical Research and the Program of the Institute:

(1) A major barrier to the effective use of liposomes as carriers in cancer chemotherapy and diagnosis has been a lack of understanding of their interactions with serum. Our studies define the interaction and indicate what type of liposomes must be used to avoid it. (2) Our studies of HDL-liposome recombinants contribute to (1) and may also be useful in delineating mechanisms of atherosclerosis. Our fluorescently labelled lipoproteins are currently being used by collaborators to study atherogenesis. (3) Breakdown of liposomes in serum is essential to the combination of liposomes with hyperthermia to achieve selective release of drugs in the area of a tumor. (4) Antigen presentation to T-lymphocytes is an important function in the immune system and identification of antigenic peptides within, for example the HTLVIII envelope polyprotein, may aid in development of vaccines.

Proposed Course:

This project has been terminated except for the work on antigenic peptides and except for the processing and publication of manuscripts.

Publications:

Klausner, R.D., Blumenthal, R., Innerarity, T., and Weinstein, J.N.: The interaction of apolipoprotein A-I with unilamellar vesicles of L-α-dipalmitoyl phosphatidylcholine. J. Biol. Chem. 260: 13719-13727, 1985.

Weinstein, J.N., Blumenthal, R., and Klausner, R.D.: Carboxyfluorescein leakage assay for lipoprotein-liposome interaction. Methods in Enzymology, in press.

Weinstein, J.N., and Leserman, L.D.: Liposomes as drug carriers in cancer chemotherapy. In I.D. Goldman (Ed.) The International Encyclopedia of Pharmacology and Therapeutics.. New York, Pergamon Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08359-05 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 charecters 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 professionel personnel below the Principal Investigetor.) (Name, title, leboretory, and institute affiliation)*

John N. Weinstein, M.D., Ph.D.	Senior Investigator	LTB, NCI
Other Professional Personnel:		
David G. Covell, Ph.D	Senior Staff Fellow	LTB, NCI
Oscar D. Holton, III, Ph.D.	Expert	LTB, NCI
Jacques Barbet, Ph.D.	Guest Worker	LTB, NCI
Mary J. Talley	Biologist	LTB, NCI
Christopher D.V. Black, Ph.D.	Visiting Fellow	LTB, NCI
Renee Eger, B.S	Guest Researcher	LTB, NCI

COOPERATING UNITS *(if any)*: Dr. A. Keenan, Dr. S.M. Larson, LHM, CC; Dr. R. Parker, Dr. S. Sieber, DCCP; Dr. R.K. Oldham, Dr. K.M. Hwang, Dr. M.E. Key, FCRF; Dr. L. Liotta, Dr. G. Bryant, LP, DCBD; Dr. J. Schlom, Dr. D. Colcher, LTIB, DCBD; Dr. M. Lotze, Dr. R. Rosenberg, SB, DCT; Dr. J. Mulshine, NCI-NMOB, DCT.

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Theoretical Immunology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.3	1.6	1.7

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.)*

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 be expected to provide higher sensitivity, lower background, lower systemic toxicity, and faster localization than the intravenous route. It will also minimize the problem of cross-reactivity with antigen present on normal tissues.

The experimental design of the guinea pig studies is currently being applied to detection of lymph node metastases in clinical stage II malignant melanoma and cutaneous T-cell lymphoma (CTCL). Similar protocols have been approved for breast carcinoma, Hodgkin's disease, 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 both to optimize the clinical procedures and to explore basic functions of the immune system (see project #Z01CB08368-2 Selective Cytotoxicity in the Lymphatics). Our longer term aim is to understand the pharmacology of monoclonal antibodies and other ligands in order to develop criteria for rational molecular design of biological antitumor agents.

Project Description:

Objectives:

(i) Establish a quantitative basis (experimental and theoretical) for lymphatic delivery of monoclonal antibody to lymph nodes; (ii) Use that information for optimization of diagnostic imaging of animal tumors; (iii) Treat the tumors with radio-labeled and/or toxin-labeled antibodies (using experimental parameters obtained from the diagnostic studies); (iv) Extend the principles of lymphatic immunodiagnosis and immunotherapy to the clinical setting; (v) Extend our theoretical and experimental models to the intravenous administration of monoclonals; (vi) Apply the lymphatic approach to detection of lymph node metastases of malignant melanoma, breast carcinoma, lymphoma, non-small cell lung carcinoma, and other malignancies; (vii) Extend the lymphatic delivery of monoclonal antibodies to therapeutic modalities; (viii) Investigate the lymphatic pharmacology of immunization and toleragenesis.

Non-Standard Methods Employed:

(a) Two-color fluorescence microscopy and fluorescence cell-sorting on cell populations labeled in vivo with monoclonal antibodies; (b) Image-correction techniques for quantitative analysis of gamma camera studies; (c) Use of SAAM modeling system to analyze the pharmacokinetics of antibody distribution and binding in vivo; (d) Filtration and centrifugation methods for determination of equilibrium and kinetic binding parameters for monoclonal antibodies; (e) Labeling of antibodies for imaging by nuclear magnetic resonance; (f) Autoradiography of diffusible antibodies; (g) Immunohistochemistry.

Major Findings:

Antibodies directed against normal cells:

(1) Antibodies against normal cell types (in the mouse and guinea pig) can be delivered with high efficiency from subcutaneous injection sites to regional lymph nodes. The normal nodes can be imaged with the gamma camera if the antibodies are radiolabeled with I-125 or I-131. (2) Injections can be made so as to label all cells of the target population in the node. (3) Pharmacokinetic experiments and modeling indicate that correct choice of dose, volume, osmolarity, and site of injection are all important if efficiency of the imaging technique is to be maximized. These findings suggest clinical use of antibodies against normal lymphoid cells as an alternative to colloid lymphoscintigraphy for diagnostic imaging of lymph nodes.

Antibodies directed against tumor cells:

(1) Antitumor antibodies can be delivered with high efficiency from subcutaneous injection sites to regional lymph nodes (in the guinea pig). (2) Early metastatic tumor in the lymph nodes is selectively labeled with the antibody, and gamma camera images of the tumor deposits can be obtained. (3) The smallest

amounts of tumor imaged in this way are about 3 mg (much smaller than has been detected by i.v. monoclonal antibodies or other non-invasive methods of detection). (3) The double-label experimental design developed for animal studies is appropriate for use in clinical trials of lymphatic delivery of monoclonal antibodies. (4) Antibodies against normal lymphoid cells can be used in tandem with antibodies against tumor. The former indicates gross characteristics of the node and/or its immunological status; the latter detects metastatic tumor with high sensitivity. (5) Subcutaneous injections of T101 antibody 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 ever 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.) and humans.

Significance to Biomedical Research and the Program of the Institute:

In the staging of metastatic tumors, it is almost always necessary to assess the status of lymph nodes. Current non-invasive techniques (standard x-ray, computerized axial tomography, lymphangiography) are usually limited in sensitivity to the detection of masses larger than 1 cubic cm. In guinea pigs we detected metastases 300 times smaller. If some portion of that sensitivity could be obtained clinically, the need for surgical assessment of nodes (e.g., in breast cancer) might be reduced. More speculatively, antibodies armed with toxins or drugs might be used to treat lymph node metastases. By a combination of in vitro cell experiments, animal studies, theoretical modeling, and clinical studies, we are exploring these possibilities.

The lymphatic route for administration of monoclonal antibodies may be useful as well for understanding and manipulating the regional immune response. These issues are significant from the point of view of sensitization and tolerance to tumors.

Proposed Course:

(1) Continue to develop systems for in vivo delivery of monoclonal antibodies to T- and B-cell sub-populations in the mouse lymph node, both for refinement of the approach to diagnosis and therapy of lymph node tumor and as a means of modulating immune function; (2) Continue to use the line 10 tumor of guinea pigs to improve strategies for clinical detection and treatment of lymph node metastases; (3) Continue study of clinical stage II melanoma and T-cell lymphoma, in collaboration with Drs. S. Larson, M. Lotze, J. Carrasquillo, A. Keenan, J. Mulshine, and others); (4) Extend the clinical studies to breast carcinoma (with Dr. J. Schlom and co-workers), small cell and non-small cell lung carcinoma (with Dr. J. Mulshine and co-workers), and Hodgkin's disease (with Dr. D. Longo and co-workers); (5) Extend the studies to therapy via immunotoxins, complement, and radioconjugates (see project #Z01CB08368-01); (6) Continue theoretical and

experimental studies of the molecular cell biology of antibody interaction with cell surface antigens and endocytic machinery; (7) Continue kinetic modeling of monoclonal antibody pharmacokinetics in animals and humans.

Publications:

Covell, D.G., Steller, M.A., Parker, R.J., and Weinstein, J.N.: Delivery of monoclonal antibodies through the lymphatics: Characterization by compartmental modeling. In: Computer Applications in Medical Care 10: 884-888, 1985.

Weinstein, J.N., Keenan, A.M., Holton, O.D., III, Covell, D.G., Sieber, S.M., Black, C.D.V., Barbet, J., and Parker, R.J.: Use of monoclonal antibodies to detect metastases of solid tumors in lymph nodes. In: Monoclonal Antibodies and Breast Cancer (R.L. Ceriani, ed.). Martinus Nijhof, Boston, 1985, pp. 218-232.

Weinstein, J.N., Parker, R.J., Holton, O.D., III, Keenan, A.M., Covell, D.G., Black, C.D.V., and Sieber, S.M.: Lymphatic delivery of monoclonal antibodies: Potential for detection and treatment of lymph node metastases. Cancer Investigation 3: 85-95, 1985.

Weinstein, J.N., Black, C.D.V., Keenan, A.M., Holton, O.D., III, Larson, S.M., Sieber, S.M., Covell, D.G., Carrasquillo, J., Barbet, J., and Parker, R.J.: Use of monoclonal antibodies for detection of lymph node metastases. In Reisfeld, R. (Ed.): Monoclonal Antibodies in Cancer Therapy, Alan R. Liss, New York, 1986, pp. 473-488.

Steller, M.A., Parker, R.J., Covell, D.G., Holton, O.D., III, Keenan, A.M., Sieber, S.M., and Weinstein, J.N.: Optimization of monoclonal antibody delivery via the lymphatics: The dose-dependence. Cancer Research 46: 1830-1834, 1986.

Weinstein, J.N., Holton, O.D., III, Black, C.D.V., Covell, D.G., Spaulding, G.F., Barbet, J., Steller, M.A., Sieber, S.M., Talley, M.J., and Parker, R.J.: Regional delivery of monocloncal antitumor antibodies: Detection and possible treatment of lymph node metastases. In Welch, D.R., Bhuyan, B.K., and Liotta, L.A., (Eds.): Cancer Metastasis: Experimental and Clinical Strategies. Alan R. Liss, N.Y. 1986, pp. 169-180.

Weinstein, John N., Parker, R., Holton, O.D. III, Black, C.D.V., Sieber, S.M., and Covell, D.G.: The pharmacology of monoclonal antibodies. In R. Reisfeld (Ed.): Membrane-mediated Cytotoxicity, Alan R. Liss, Inc., New York, in press.

Weinstein, John N.: Immunolymphoscintigraphy and other regional applications of monoclonal antibodies. In: M. Zalutsky (Ed.): Antibodies in Radiodiagnosis and Therapy, CRC Press, Boca Raton, Fla, in press.

Weinstein, John N., Black, C.D.V., Parker, R., Eger, R., Sieber, S.M., Mozayeni, B., and Covell, D.G.: Quantitative aspects of the pharmacology of monoclonal antibodies. Site-specific Drug Delivery: Cell Biology, Medicinal and Pharmaceutical Aspects, in press.

Lotze, M.T., Carrasquillo, J.A., Weinstein, J.N., Bryant, G.J., Perentesis, P., Reynolds, J.C., Matis, L.A., Eger, R.R., Keenan, A.M., Hellstrom, I., Hellstrom, K.-E., and Larson, S.M.: Monoclonal antibody imaging of human melanoma: Radioimmunodetection by subcutaneous or systemic injection, Annals of Surgery, in press.

Covell, D.G., Barbet, J., Holton, O.D. III, Black, C.D.V., and Weinstein, J.N.: The pharmacokinetics of monoclonal IgG1, $F(ab')_2$ and Fab' in mice. Cancer Research, in press.

Keenan, A.M., Weinstein, J.N., Mulshine, J.L., Carrasquillo, J.A., Bunn, P.A., Jr., Reynolds, J.C., Foon, K.A., Perentesis, P., Ghosh, B., and Larson, S.M.: Evaluation of lymphoma by immunolymphoscintigraphy: Subcutaneous injection of indium-111-labeled T101 monoclonal antibody. J. Nucl. Med., in press.

Covell, D.G., Barbet, J., Holton, O.D. III, Black, C.V.D., Keenan, A.M., Sieber, S.M., and Weinstein, J.N.: Global and local factors in the pharmacology of monoclonal antibodies. American Statistical Society Symposium, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08363-04 LTB

PERIOD COVERED: October 1, 1985, to September 30, 1986

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

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

H. Robert Guy, Ph.D.	Expert	LTB, NCI

Other Professional Personnel:

Robert Jernigan, Ph.D.	Theoretical Physical Chemist	LTB, NCI

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	1.2	0.1

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 primary goal of this project is to develop methods to predict structures of membrane proteins from their sequences and available experimental data. We have then applied these methods to develop structural models of several membrane proteins.

Many protein sequences were analyzed with methods previously developed to predict which portions of α helices and β strands should be exposed to water, other protein segments, or buried in membranes among hydrocarbon lipid chains. Molecular models of the action potential channel, δ hemolysin, and voltage dependent anion channel (VDAC) of mitochondrial outer membrane were developed by analyzing their sequences using this and other methods.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Major Findings

The original goal of developing methods to predict structures of transmembrane portions of membrane channel proteins and of using these methods to construct three dimensional models have been realized for the action potential sodium channel, δ lysin, and VDAC.

The sodium channel is the most complex protein we have attempted to model. It is also the most complete and satisfying model we have developed because it not only describes the general structure of the transmembrane region, but also describes how the channel is opened and closed by changing the membrane voltage and identifies atoms responsible for making the channel selective for sodium. All essential features of this model are conserved in rat and eel sequences whereas many non-essential segments or regions are not well conserved. This finding strongly supports the model since it was developed from the eel sequence alone with no way of predicting which segments would be conserved in other species. In developing this model, an attempt was made to design a structure that contains features which have been proposed on the basis of voltage clamp studies of sodium channels. Dimensions of the narrowest region of the channel in the modified model and nature of the atoms that form this region are very similar to the model of the "selectivity filter" proposed by Hille on the basis of permeability of the channel to organic cations. Dimensions and choice of side chains that form this region were made completely on the basis of packing adjacent α helices with no attempt to make the structure conform to Hille's model. Activation kinetics of channel opening have been studied extensively. These studies indicate that a series of voltage dependent conformational changes occur before the channel opens. The model postulates how these changes occur and the most recent version of the model postulates how differences between the last domain and the other domains are consistent with kinetic schemes proposed by Armstrong and associates. After the channel opens, it can inactivate or close. Inactivation can be modified from the cytoplasmic side by proteolytic treatment, chemical modification, or by several toxins. It can also be modified from the outside by scorpion toxins that bind in a voltage dependent manner. Armstrong has postulated that inactivation is caused in part by a positively charged segment on the cytoplasmic side of the membrane that moves into the channel entrance after the channel opens. We have postulated which segment forms this "inactivation plug" and how inactivation is modulated from the outer surface by scorpion toxins. The model predicts how changing the sequence by site-directed mutagenesis should effect measurable properties.

The simplest membrane protein we have been analyzing is δ lysin, a lytic toxin from staphylococcus. We have selected δ lysin because it is only 26 residues long and because analyses of its sequence suggest it can form amphipathic α helices with properties similar to those of putative amphipathic α helices that form the channel lining in more complex proteins we have modeled. We have used the Empirical Conformation Energy Program for Peptides (ECEPP) to calculate relative energies of alternative conformations of the δ lysin monomer. These

calculations predict an α helical conformation for fourteen residues in the middle of the sequence. These results are consistent with experiments that indicate its α helical content, and its dependence on the solvent. We have developed a method to add effects of water and lipid environments to the detailed interatomic energy calculations. This method is being used to study how δ lysin monomers aggregate in water and in membranes.

The sequence of the VDAC channel from the outer membrane of yeast mitochondria has been determined by two groups. Structural models of this channel are being developed in collaboration with H.R. Guy and Michael Forte. These studies indicate that, unlike the proteins described above, VDAC has no segments that can form transmembrane hydrophobic or amphipathic α helices. It does have up to nineteen segments that could cross the membrane as β strands if one side of each strand can be exposed to water inside the channel while the other side is in an hydrophobic lipid or protein environment. We have developed a tentative model in which these putative β strands form a large β barrel structure with a pore of about 24 angstroms diameter through its center. This model is consistent with electron microscopy and other structural studies.

Publications

Guy, H.R.: "Review and Revision of Structural Models for the Transmembrane Portion of the Acetylcholine Receptor Channel" Proceedings of the NATO Advanced Workshop on mechanism of action of the nicotinic acetylcholine receptor, in press.

Wilson, S.H., Cobianchi, F., and Guy, H.R.: cDNA cloning and structural for relation of a mammalian helix destabilizing protein snRNP particle core protein A1. In Thompson, E.B. and Papaconstantinou, J. (Eds.): DNA: Protein Interactions and Gene Regulation. Houston, Univ. of Texas Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08365-04 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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. — IPA Investigator — 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, Director for Health & Environmental Research, U.S. Dept. of Energy

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

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.)*

We have developed an algorithm to predict protein antigenic sites recognized by T lymphocytes. The approach is based on the finding that a majority of the T-cell sites tend to form amphipathic α-helices. We have optimized an algorithm which uses a power spectrum based on a least squares fit to a sine wave. By applying this procedure to a sequence of hydrophobicity values derived from the amino acid sequence, we detect amphipathic helical segments along the protein, corresponding to T-cell sites. These results reveal fundamental aspects of the chemistry of T-cell recognition, and may be very useful for predicting sites for the development of synthetic peptide vaccines.

Project Description

Objectives:

To develop an optimized algorithm for predicting amphipathic helical T cell antigenic sites from the amino acid sequence of a protein.

Methods Employed:

Computational, statistical, physical - chemical analysis

Major Findings:

We have used a data base of 23 known helper T cell sites, located on 12 proteins, to systematically develop an optimized algorithm, for predicting T cell antigenic sites based on the amphipathic helix model. The choice of power spectrum procedure, hydrophobicity scale and model parameters were examined. Algorithms were tested by comparing the predicted amphipathic segments with the locations of the known T cell sites and calculating the probability of getting this number of matches by chance alone. The optimum algorithm, which predicts the largest number of sites with the lowest chance probability, uses the Fauchere-Pliska hydrophobicity scale and a least squares fit to a sine wave as its power spectrum procedure. By applying this algotithm 18 of the 23 sites are predicted (75% sensitivity) with a high degree of significance ($p<0.001$). These findings support the hypothesis that stable amphipathic structures such as an amphipathic helix are fundamentally important in determining immunodominance.

Significance to Biomedical Research and the Program of the Institute:

Starting with the primary amino acid sequence of a foreign protein in which, theoretically, every subsequence of residues along the protein might be antigenic, the algorithm narrows considerably the number of canidate sites directing the experimental efforts towards the most promising segments. This approach will be of great practical value in designing synthetic vaccines aimed at T cell immunity.

Proposed Course:

Application of this method to antigens of clinical importance such as the AIDS virus, malaria and leprosy are in progress. We also plan to develop other methods to predict secondary structure of proteins and peptides and study other structural features that are useful in predicting antigenic sites.

Publications:

DeLisi, C., Cornette, J. L., Margalit, H., Cease, K., Spouge, J. L., and Berzofsky, J. A.: The role of amphipathicity as an indicator of T-cell antigenic sites on proteins. In: Secarz, E.E. and Berzofsky, J.W. (Eds): Immunogeneicity of Protein Antigens: Repertoire and Regulation. CRC press, in press.

Berzofsky, J. A., Cornette, J. L., Margalit, H., Berkower, I., Cease, K., and DeLisi, C.: Molecular features of class II MHC-restricted T-cell recognition of protein and peptide antigens: The importance of amphipathic structures. In Melchers, F., and Koproski, H. (Eds).: Current Topics in Microbiology and Immunology. Springer-Verlag, in press.

Berzofsky, J. A., Berkower, I. J., Cease, K. B., Margalit, H., Cornete, J. L., Spouge, J. L., Spencer, C., Buckenmeyer, G., Streicher, H., Kojima, M., and DeLisi, C.: The role of MHC and amphipathic structures in T cell recognition: Features determining immunodominance. In Vogel, H., Pernis, B., and Silverstein, S. (Eds.): Processing and Presentation of Antigens, Columbia University Press, N.Y., in press.

Berzofsky, J. A., Cease, K. B., Berkower, I. J., Margalit, H., Cornette, J. L., and DeLisi, C.: Immunodominance of amphipathic peptides and their localization on the cell surface for antigen presentation to helper T cells. In Cinader, B. and Miller, R. G. (Eds.): Progress in Immunology VI, (Sixth International Congress of Immunology, Toronto, July, 1986), Academic Press, N.Y., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08366-03 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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 professionel personnel below the Principal Investigetor.) (Name, title, laboratory, and institute affiliation)*

John N. Weinstein, M.D., Ph.D.	Senior Investigator	LTB, NCI
Other Professional Personnel:		
David G. Covell, Ph.D	Senior Staff Fellow	LTB, NCI
Jacques Barbet, Ph.D.	Guest Researcher	LTB, NCI

COOPERATING UNITS *(if any)*: Dr. L. Liotta, LP, DCBD; Dr. S.M. Larson, NM, CC; Dr. B. Bunow, LAS, DCRT

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Theoretical Immunology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

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

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 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 antibodies, lymphokines, and other growth factors.

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

Project Description

Objectives:

(1) To investigate factors which limit the access of monoclonal antibodies and other ligands to target cells within tumors; (2) To investigate analogously limited access of physiological ligands (e.g., growth factors) to cells within tumors.

Non-standard Methods Employed:

(1) Fluorescence-labeling and radio-labeling of monoclonal antibodies; (2) Double-label autoradiography of diffusible substances; (3) Computer simulation of convection-diffusion equations with non-linear binding, using the PDE-COL program; (4) Use of the SAAM compartmental modeling system to determine boundary conditions for distributed models; (5) In vitro growth of small cell lung carcinoma as spheroids; (6) Intravenous metastasis model in nude mice.

Major Findings:

(1) For physiologically reasonable ranges of parameters, the diffusion coefficient and/or hydraulic conductivity may limit flux of antitumor antibodies through tumors; (2) The flux of non-binding control antibody is much less likely to be limited by the rate of diffusion or convection through the tumor. Such antibodies are predicted to penetrate more deeply and more quickly into the tumor; (3) In the presence of saturable binding but not metabolism, the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. However, the period of exposure will be later for cells farther from the source of antibody; (4) Metabolism will decrease the relative CxT exposure of cells farther from the source. This may be a major barrier to effective treatment of solid tumors with ligand molecules; (5) Antibodies of low affinity may be preferable to those of high affinity for some purposes, e.g. in therapy with alpha-emitting conjugates or toxin conjugates.

Significance to Biomedical Research and the Program of the Institute:

(1) For rational planning of diagnostic imaging procedures with monoclonal antibodies and other ligands, it is necessary to investigate the spatial and temporal profiles created by diffusion, convection, and binding; (2) For planning of therapy with ligands, it is, likewise, important to understand the way in which the molecule distributes throughout a tumor; (3) In order to understand the function of endogenous biological ligands with respect to established tumors, it is likewise necessary to know how the ligand molecules distribute; (4) The finding that high affinity is not always best may change the basis for choosing which monoclonal antibodies to select for use from a hybridization. It may also influence the design of new "immunoreactive" agents by recombinant techniques.

Proposed Course:

(1) Continue theoretical studies of the convection and diffusion of antibodies into tumors, with saturable binding and metabolism included; (2) Incorporate more complex binding kinetics (for monovalent vs. divalent binding) to compare the characteristics of IgM, IgG, $F(ab')_2$, and Fab; (3) Extend the studies to include the simultaneous presence of competing ligands; (4) Do correlative experiments in vivo using i.v. injection of human melanoma cells to develop lung and liver metastases in nude mice. The mice will be injected with I-131-labeled antitumor antibody and I-125-labeled control antibody for determination of the distribution by double-label autoradiography. Analogous experiments will be done with fluorescently labeled antibody. (5) A second experimental collaboration has been established, with Dr. R. Jain of the Carnegie-Mellon Institution.

Publications:

Weinstein, J.N., Parker, R.J., Holton, O.D., III, Black, C.D.V., Sieber, S.M., and Covell, D.G.: The pharmacology of monoclonal antibodies. In Reisfeld, R. (Ed.): Membrane-Mediated Cytotoxicity, Alan R. Liss, Inc., New York, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08367-03 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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

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

Christopher D.V. Black, Ph.D., Visiting Fellow, LTB, NCI

Other Professional Personnel:

Jacques Barbet, Ph.D., Guest Researcher, LTB, NCI
O. Dile Holton, III, Ph.D., Expert, LTB, NCI
John N. Weinstein, M.D., Ph.D., Senior Investigator, LTB, NCI

COOPERATING UNITS *(if any)*

Drs. R.J. Parker and S.M. Sieber., OD, DCE, NCI; Dr. O.A. Gansow and R.W. Atcher., COP, DCT, NCI; Drs. R.A. Kroczek and E.M. Shevach., LI, NIAID

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Theoretical Immunology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 1.0	OTHER: 0.2

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.)*

Following subcutaneous injection, radiolabeled monoclonal antibodies bind efficiently to normal and tumor target cells in the lymph nodes (Project # Z01 CB 08359-02 LTB). This finding prompted us to attempt specific therapy using monoclonal antibodies covalently coupled to plant toxins. The first development along these lines has been to synthesize monoclonal antibody-ricin A-chain conjugates using four antibodies of different specificities. We then demonstrated the capacity of these conjugates to bind to their target cells and to inhibit protein synthesis at the ribosomal level in an acellular system. Unfortunately, using the guinea pig hepatocarcinoma cell line (L 10), which expresses large quantities of target antigen, we found only weak cytotoxicity with the monoclonal antibody D3 coupled to ricin A-chain.

However, the same toxin coupled to an anti-mouse MHC antibody has proved to be highly toxic for lymphoid cells and similar results have been found with ricin A-chain conjugated to monoclonal antibodies against subsets of mouse lymphocytes in vitro. We have also been able to augment the effects of these toxins by the action of certain drugs which are known to affect cell biological processes. 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 modality for specific cell killing in vivo is to use radioactive compounds attached to antibodies which, on binding to cell surfaces, can damage the cells by radiation. We have been able to demonstrate that lymph node cell ablation occurs in mice injected subcutaneously with an anti-murine B cell antibody labelled with the alpha particle emitter bismuth 212. Studies are in progress to optimize this system and to reduce the non-specific toxicity.

Project Description

Objectives:

To investigate the ability of monoclonal antibodies to kill specific cells within the lymphatic system. More specifically:

1) To couple monoclonal antibodies chemically to plant toxins, principally ricin or its catalytic sub-unit, and to determine whether this chemical modification has altered the binding or toxic properties of the constituents;

2) To determine, in vitro, the selectivity and cytotoxic efficacy of these immunotoxins against tumor cells and the cellular constituents of the immune system;

3) To determine, in vivo, the selectivity and cytotoxic efficacy of these immunotoxins against tumor cells and the normal cellular constituents of the lymphatic system;

4) Using mitogens or allogeneic cells, to investigate the in vivo stimulation of the cellular constituents of the lymphatic system with special reference to the induction of the IL-2 receptor.

5) To use monoclonal antibodies in conjunction with complement to lyse selected target cells both in vitro and in vivo in the lymph nodes;

6) To use monoclonal antibodies coupled to radioisotopes for selective killing of tumor cells or normal cells in the lymphatic system;

7) To compare the pharmacokinetic behavior of immunotoxins with that of the native monoclonal antibody (see project # Z01 CB 08359-02 LTB);

8) To use the methods developed under objectives 1-7 to design rational chemotherapeutic modalities for the subcutaneous treatment of metastatic cells in the lymph nodes;

9) Similarly, to use the information obtained above to modify regional immune responses by selective ablation of lymph node cells.

10) To test further the "neutral bypass" hypothesis for entry of toxin A-chain moieties into the cytoplasm of B-lymphocytes after attachment of toxin/antibody conjugates to class I MHC determinants.

11) To test extension of the hypothesis to other antigens, cell types, and toxic agents.

Non-Standard Methods Employed:

a) The preparation of antibody-toxin conjugates using the heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio)proprionate (SPDP).

b) Investigation of the effects of immunotoxins on cell viability using radiotracer techniques and fluorescent reagents.

c) Fluorescent immunochemical staining and flow cytofluorometric analysis.

d) Use of the SAAM modeling system to analyze the pharmacokinetics of antibody and immunotoxin distribution and binding in vivo.

Major Findings:

1): Covalent coupling of ricin A-chain to monoclonal antibodies with the SPDP reagent has been obtained using 4 antibodies of different specificities. In each case, there has been little detectable loss of antibody affinity following the coupling reaction. In addition, the ability of the A-chain of ricin to inhibit protein synthesis at the ribosomal level is not significantly impaired.

2): Much of the initial work has been concerned with the monoclonal antibody D3 which recognizes the guinea pig hepatocarcinoma cell line L10. We have been unable to show any specific toxicity due to the D3-Ricin A-chain conjugate against L10 cells in vitro despite the fact that the immunotoxin binds to these cells with an affinity very similar to that of the native antibody and despite the fact that the conjugate is still able to inhibit protein synthesis in an acellular assay. Closer examination of L 10 cells showed that they lose some of the antigen recognised by D3 when they are removed from the animal and put into culture. In addition, fluorescence microscopy revealed that these cells are only poorly able to internalize either D3 alone or the D3-ricin A-chain conjugate. The antibody-antigen complex on these cells appear to be capped and then shed into the medium. Such activity could well account for the low reactivity of the D3-ricin A-chain immunotoxin.

3): Immunotoxins having specificities for mouse lymphocyte sub-populations have also been prepared. These reagents have been tested to determine their reactivities to target cells in vitro and have proved to be highly specific and toxic to the appropriate cell type. Drugs which are known to affect intracellular traffic (eg: ammonium chloride, nigericin) have also been used to augment the effects of these immunotoxins in vitro. 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): Experiments to determine the biological distribution of the uncoupled antibodies following administration via the lymphatic system are described in another report (Project # Z01 CB08359-02 LTB). These experiments must precede the in vivo use of immunotoxins and of antibody/complement.

Significance to Biomedical Research and to the Program of the Institute:

The objectives of this project relate to the possible therapeutic and diagnostic uses of monoclonal antibodies . This particular project may be considered as the chemotherapeutic arm of the on-going investigations of this group into the delivery of biological agents to the lymphatic system by subcutaneous injection. Previously we have demonstrated substantial targeting of monoclonal antibodies to

the lymph nodes. This has been used to detect metastatic cells in the nodes of animals and has led to several clinical protocols. The present project is intended to follow up this work by producing cytotoxic systems directed to specific cell types within the nodes. In addition to the chemotherapeutic uses of these systems against cancer metastases, specific cell ablation in the lymph nodes may offer insights into the balance between regional and global control of the immune system and into the ways in which the immune responses may be usefully modified. Substantiation and generalization of the "neutral bypass" hypothesis would have considerable impact on the way in which toxin conjugates of antibody are designed.

Proposed Course:

Each of the objectives proposed above will be pursued:

#1: We will investigate three separate questions which require clarification before immunotoxins can be usefully employed as chemotherapeutic agents in vivo.

a): The stability of the conjugates when in contact with the biological milieu.

b): The ability of these molecules to enter the lymphatic system and from there to penetrate into target tissues such as the interior of tumor masses or lymph nodes;

c): The selectivity and efficiency of these cytotoxic systems for their targets in vivo.

#2: Immunotoxins and immunoglobulin/complement techniques will be used for selective killing of metastatic tumor cells in the lymph nodes.

#3: Radiolabelled immunoglobulins will be used for selective killing of metastatic tumor cells in the lymph nodes.

#4: Analogous techniques will be used in attempts at selective ablation of immunologically significant cell subsets in the lymph nodes.

Publications:

Covell, D.G., Steller, M.A., Parker, R.J., and Weinstein, J.N.: Delivery of monoclonal antibodies through the lymphatics: Characterization by compartmental modeling. In: Computer Applications in Medical Care 10: 884-888, 1985.

Weinstein, J.N., Keenan, A.M., Holton, O.D., III, Covell, D.G., Sieber, S.M., Black, C.D.V., Barbet, J., and Parker, R.J.: Use of monoclonal antibodies to detect metastases of solid tumors in lymph nodes. In Ceriani, R.L. (Ed.). Monoclonal Antibodies and Breast Cancer Boston, Martinus Nijhof, 1985, pp. 218-232.

Weinstein, J.N., Parker, R.J., Holton, O.D., III, Keenan, A.M., Covell, D.G., Black, C.D.V., and Sieber, S.M.: Lymphatic delivery of monoclonal antibodies: Potential for detection and treatment of lymph node metastases. Cancer Investigation 3: 85-95, 1985.

Weinstein, J.N., Black, C.D.V., Keenan, A.M., Holton, O.D., III, Larson, S.M., Sieber, S.M., Covell, D.G., Carrasquillo, J., Barbet, J., and Parker, R.J.: Use of monoclonal antibodies for detection of lymph node metastases. In Reisfeld, R. (Ed.): Monoclonal Antibodies in Cancer Therapy, Alan R. Liss, New York, 1986, pp. 473-488.

Steller, M.A., Parker, R.J., Covell, D.G., Holton, O.D., III, Keenan, A.M., Sieber, S.M., and Weinstein, J.N.: Optimization of monoclonal antibody delivery via the lymphatics: The dose-dependence. Cancer Research 46: 1830-1834, 1986.

Weinstein, J.N., Holton, O.D., III, Black, C.D.V., Covell, D.G., Spaulding, G.F., Barbet, J., Steller, M.A., Sieber, S.M., Talley, M.J., and Parker, R.J.: Regional delivery of monocloncal antitumor antibodies: Detection and possible treatment of lymph node metastases. In Welch, D.R., Bhuyan, B.K., and Liotta, L.A., (Eds.): Cancer Metastasis: Experimental and Clinical Strategies. Alan R. Liss, N.Y. 1986, pp. 169-180.

Weinstein, J.N., Parker, R., Holton, O.D. III, Black, C.D.V., Sieber, S.M., and Covell, D.G.: The pharmacology of monoclonal antibodies. In R. Reisfeld (Ed.): Membrane-mediated Cytotoxicity, Alan R. Liss, Inc., New York, in press.

Covell, D.G., Barbet, J., Holton, O.D. III, Black, C.D.V., and Weinstein, J.N.: The pharmacokinetics of monoclonal IgG1, $F(ab')_2$ and Fab' in mice. Cancer Research, in press.

Covell, D.G., Barbet, J., Holton, O.D. III, Black, C.V.D., Keenan, A.M., Sieber, S.M., and Weinstein, J.N.: Global and local factors in the pharmacology of monoclonal antibodies. American Statistical Society Symposium, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08368-03 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: A Mathematical Model of Nonspecific Monclonal Antibody Pharmacology

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

David G. Covell, Ph.D., Senior Staff Fellow — LTB, NCI

Other Professional Personnel:

John N. Weinstein, M.D., Ph.D., Senior Investigator — LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 0.25	PROFESSIONAL: 0.25	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Monoclonal antibodies or other ligands are potentially useful for the diagnosis and treatment of tumors. Their potential depends on the ability of the antibody to reach the target cell. We have developed a theoretical model for the biodistribution of nonspecific whole monoclonal antibody and the FAB_2' and FAB' fragments following intravenous injection. The model incorporates processes for transcapillary transport of antibody via diffusive and convective pathways, entry of antibody into the lymphatics system and metabolic degradation of antibody.

Significant theoretical findings include the following: whole IgG a) remains in the body much longer than its fragments, b) has a distribution volume exceeding that of plasma plus interstitial fluid, c) distributes into these volumes rapidly for most enteral organs and slowly for muscle, d) produces interstitial:plasma concentration ratios > 0.5 for enteral organs and 0.18 for carcass, e) has the greatest percentage of its catabolism due to gut, and f) cycles through the interstitial spaces at least 2.8 times/gram organ weight before being metabolized or excreted. When compared with whole IgG, the Fab' fragment a) is cleared from the body much faster, b) has a larger total distribution volume, c) distributes more rapidly into this volume d) produces higher interstitial:plasma concentration ratios, e) is catabolized principally by the kidney, followed by the gut, then the spleen, and f) cycles through non-kidney interstitial spaces at least 0.4 cycles/gram tissue weight before metabolism or excretion. The $F(ab')_2$ fragment has pharmacokinetic characteristics that fall between those of whole IgG and Fab'.

These results 1) provide pharmacokinetic criteria for selecting whole IgG, $F(ab')_2$ or Fab' for various *in vivo* applications, 2) provide a framework for predicting cumulative tissue exposure to antibody labeled with different isotopes, and 3) provide a reference metabolic state for the analysis of more complex systems that do include antibody binding.

GPO 914-916

Project Description

Objectives:

To investigate factors that improve the targeting of intravenously administered monoclonal antibodies.

Nonstandard Methods Employed:

(1) Computer simulation of ordinary and partial differential equations describing the theoretical model.

Major Findings:

1) Intravenously administered antibody fragments are catabolized and excreted much faster than whole antibody and as such produce lower cumulative tissue exposure.

2) Significant clinical trade-offs can be expected when using rapidly cleared antibody fragments. The smallest fragment, with the fastest clearance, also cycles the fewest times through regions in the body where tumor may exist. This characteristic may prove to offset any advantages in localization due to lower background exposure.

3) The proposed model provides a basis for analysis of antibody pharmacology in systems that include binding to tumor and circulating antigen.

Significance to Biomedical Research and the Program of the Institute:

(1) Monoclonal antibodies or other ligands are potentially useful for the diagnosis and treatment of tumors in lymph nodes. Their therapeutic and diagnostic potential depends on the ability of the antibody to reach the target cell. (2) To improve delivery of monoclonal antibodies to target cells it is important to understand how molecules are distributed throughout body tissues.

Proposed Course:

(1) Continue theoretical studies on the movement of macromolecules from tissues into the capillary and lymphatic systems. (2) To apply the results of this analysis to a more complex model describing the distribution kinetics of monoclonal antibodies in nodal tissue. (3) To apply these models to the design and analysis of animal experiments and human trials.

Publications:

Covell, D.G., Steller, M.A., Parker, R.J., and Weinstein, J.N.: Delivery of monoclonal antibodies through the lymphatics: Characterization by compartmental modeling. In: Computer Applications in Medical Care, 10, I.E.E.E. Press, pp. 884-888, 1985.

Covell, D.G., Barbet, J., Holton, O.D. III, Black, C.D.V., and Weinstein, J.N.: The pharmacokinetics of monoclonal IgG1, $F(ab')_2$ and Fab' in mice. Cancer Research, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08369-03 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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	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 objective is to functionally unite the wide variety of image processing equipment working in the laboratory so that the results of procedures performed on one processor may be available to the user at any other component. To this end two major software packages have been specified, designed, implemented and largely debugged. These are 1) BMIO, a basic set of input output routines which provide for interprocessor transfer of generalized digitized images, and 2) SPIDER a data independent context free packet switching network in which our DEC System 20 is the permanent master.

Project Description:

Objectives:

Our long term objective is to maximize the impact of our work on the protein analysis and nucleic acid structure research communities, and also to maximize the utility of the recently acquired hardware image processing systems (Comtal) in anticipation of the impending obsolesence of the PDP/8- quantimet predecessor. Accordingly we have devoted much time and effort to the problem of system export, first with Gellab) the problem of production nucleic acid secondary structure calculation for gene sized sequences (a dedicated 68000 class processor(s) is essential) and lastly the construction of system software to allow control of all significant hardware via the System-20, the most "intelligent" of our processors. The system software involves construction of two components, BMIO and Spider, which are detailed below. The work on BMIO included the design specification, design document, programming, testing,and user document. The function of BMIO is to allow simple, flexible access to a variety of I/O devices (the RTPP's and disks) with data consisting of picture files in a multitude of data formats (1, 2, or 4 pixels per word), (picture, boundary, or other descriptive information), and picture sizes (256x256, 512x512, 256x4096, 4096x256, etc.). This work required the development of a user-controlled paging facility on the DECSYSTEM-2020. Once developed, many of the facilities of BMIO were made use of in other areas, such as the use of the paging system and of the disk access system in the nucleic acid secondary synthesis programs.

Methods Employed:

The function of SPIDER is to provide a simple, flexible mechanism for data-independant context-free packet switching among the DECSYSTEM-2020 and a variety of peripheral devices such as the COMTAL VISION ONE/20 image processing systems. An interface between the DECSYSTEM-2020 and the COMTALs is provided by a DEC PDP 11 used as a packet switcher in a modified wheel local network arrangement whereby the PDP 11 is the central hub of the network and all packets must go through it. However all devices hung off spokes of the wheel are not of equal status. The DECSYSTEM-2020 is always the master and whichever other devices are talking with it are always treated as slaves. In order to implement the network several things were needed. A device driver was needed for the PDP 11 to enable it to talk to the various devices attached to the network (the DECSYSTEM-2020 and the COMTALs). A set of packet encoder/decoder modules were needed for the PDP 11 to translate data and commands into a form suitable for the COMTALS. A network communications device handler was needed for the DECSYSTEM-2020 to enable it to talk to the PDP 11. A set of packet encoder/decoder modules were needed for the DECSYSTEM-2020 to translate data and commands from the user programs into a form suitable for the common network communications format. A set of test programs were needed to test and facilitate debugging of the implementation of the network.

Major Findings:

Implicit in the design of the packet encoder/decoder modules was a solid knowledge of the facilities provided by the COMTALs and how to gain access to them from a program residing on the PDP 11. In addition, in order to facilitate the implementation of the network although not absolutely necessary to the network we designed and coded a program residing on the DECSYSTEM-2020 which allows access to both the disk file system of the PDP 11 and its terminal system, including the control console terminal. This allowed the down-loading of programs from the DECSYSTEM-2020 to the PDP 11 and their transfer back to the 2020 for further development. It also provided for automatic bootstrap loading of the PDP 11 upon command from the 2020. This allows for true mastery of the network to be maintained in one central location, on the 2020. This program has since been modified to allow the 2020 to communicate with a variety of other computers, and is currently being used by Dr. Bruce Shapiro to communicate with the several DEC VAXes in the Laboratory of Mathematical Biology.

Significance to Biomedical Research and the Program of the Institute:

It makes available to both on site and remote users within the Institute the complex of software and firmware facilities largely unique to the IPS. Thus image processing techniques can now be widely applied to the solution of problems of protein and nucleic acid structure and function.

Proposed Course:

Our long term objective is to maximize the impact of our work on the protein analysis research communities, and also to maximize the utility of the recently acquired hardware image processing systems (COMTAL) in anticipation of the impending obsolesence of the PDP/8-RTPP predecessor. Accordingly we have devoted much time and effort to the problem of system export and upgrade to easily maintainable systems.

With the computation and storage requirements for the future microGELLAB a dedicated microVAX/68000/16032 class processor is essential and work in the area of system analysis has progressed. Secondly, the construction of IPS SPIDER network software allows control of all significant hardware connected to our DECsystem-10 processor. Additional work on SPIDER now permits simultaneous device access as well as enhancements on SPITST (a maintenance software tool).

1. The midiC C compiler was completed to the extent that it was usable enough to test, on our DEC10, concepts for the conversion of the GELLAB SAIL code to C. When nearly complete, we purchased a newly released C compiler for the DEC10 - the SARGASSO C compiler.

2. The major effort shifted to the conversion of the GELLAB SAIL code to C. PSAIL is a more complete SAIL to C translator than the original SAITOC program (started last year) and will hopefully enable us to export SAIL programs previously developed on our DEC10 to other processors of the VAX/68000/16032 class.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08370-03 LTB

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters 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

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:
0.3	0.2	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.)*

Effective residue-residue interaction energies have been statistically derived from protein X-ray structures. A lattice-like model is used in which each residue type has a coordination number. If a specific residue has an incompletely filled coordination shell, then it is assumed to be filled with equivalent water molecules. Derived contact energies follow intuition: The most favorably interacting pairs are hydrophobic residues. However, those interactions are quite non-specific. More specificity is observed between polar residues. Protein folding schemes based on such energies favoring hydrophobic interactions are being developed. These involve developing methods to generate compact, dense conformations in more efficient ways. Simple lattice models of protein folding are being considered. Dynamic programming schemes are being developed to determine which of the possible strongest interactions are consistent with sequence and steric constraints.

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

Project Description:

Objectives:

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. However, complexities of molecular environments make it difficult to quantitatively represent, with a single calculation method, the full range of situations in which a macromolecule may exist. 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.

Methods Employed:

Thorough treatments of protein conformations require including solvent interactions with the protein. This is a problem that has not yet been treated satisfactorily. Solvent can interact with a protein most obviously by forming hydrogen bonds with appropriate atoms on its surface and affecting the stability of a conformation by the extent to which the protein "disturbs" the structure of the surrounding water. In addition, electric interactions inside a protein could be affected by solvent. So-called "hydrophobic" interactions may be the principal solvent effect. They do not necessarily depend on specific details of the solvent arrangements. However, they essentially represent an attempt of the hydrocarbon portions of a protein to bury themselves and thus avoid their unfavorable interactions with water. These effects play a major role in protein folding.

We have estimated the contact energies between residues in proteins directly from the observed numbers of residue-residue contacts in crystal structures. Our model is a lattice occupied by either residues or "effective solvent molecules" that are composed of a group of actual solvent molecules of total size equal to that of an average residue. Interactions occur only between nearest neighbors. Each type of residue has a different size and slightly different number of neighbors within a radius R here taken as 6.5 A. Neighboring residues along the sequence have been excluded in the counting. We count the numbers n_{ij} of all pairs of contacts between different types of residues i and j. The total contact energy is simply the sum of the energies of contact pairs:

$$E = \Sigma \Sigma E_{ij} n_{ij},$$

where E_{ij} is the energy of contact between the ith and jth types of residues. Two types of energies can be derived. First, consider the exchange of contacts

$$i—j + 0—0 \rightleftarrows i—0 + j—0$$

where i and j are types of residues and 0 is solvent. The energy for this exchange is

$$e_{ij} = E_{ij} + E_{00} - E_{i0} - E_{j0}$$

We can derive this type of relative energies from the crystal structures by the application of molecular theory. These energies are directly related to the numbers of pairs by equilibrium expressions:

$$\exp(-e_{ij}) = \frac{n_{ij}\, n_{00}}{n_{i0}\, n_{j0}}$$

where n_{ij} is the average number of ij contact pairs. We require the number of effective solvent molecules n_0. This will be estimated from the possible interactions of a random coil with solvent. Such an estimate will reflect the effects of chain connection since the chain connection does not permit each residue to be completely surrounded by solvent. The denatured state can be viewed as a cylindrical worm surrounded by solvent. This description of the denatured state is theoretically difficult, but it is chosen here as the state for which $e_{ij} = 0$. The number of equivalent solvent molecules is found to be on average 2.3 per residue for 200 residues. Since the average coordination number is 6.3, it is clear that these denatured proteins are still compact, with large numbers of inter-residue contacts.

The numbers of solvent contacts, n_{i0} and n_{j0} are found by completing the coordinate spheres around each residue; the n_{00} can then be derived if the total number of solvent molecules n_0 is known.

The average e_i is defined as

$$e_i = \frac{\Sigma\, e_{ij}\, n_{ij}}{n_{ir}}$$

The latter e_i times the coordination number of the ith type of residue is similar to a hydrophobicity scale. Comparison with the Nozaki-Tanford hydrophobicity scale indicates good agreement. In a recent thorough assessment of hydrophobicity scales by Cornette, Cease, Margalit, Spouge, Berzofsky and DeLisi for purposes of discriminating amphipathic conformations, this scale proved to be best among the theoretically derived scales.

Major Findings:

The following features are notable in the pairwise energies: The largest favorable energies e_{ij} are among the non-polar residues. This type of energy has a direct bearing on folding. Other notable features are CYS-X formation from CYS-CYS pairs corresponds to a large loss of energy because of the frequent occurrence of CYS-CYS pairs. From energies for pairs relative to self-interactions, it is seen that opposite charge pairs (- GLU, - ASP AND + LYS, + ARG) are more favorable than contacts between charge pairs of the same sign. There is a segregation of hydrophobic and hydrophilic residues. For hydrophobic residues these energies are small, indicating no strong preferences among one another, i.e. they are relatively randomly mixed; whereas the large positive values exhibited between hydrophobic and hydrophilic residues indicate their segregation. Most other features of these energies are expected. The values reflect the actual situation inside proteins. These energies provide a tool that can be applied to a variety of problems and, in particular, can be used to assess the relative stabilities of different conformations.

Significance to Biomedical Research and the Program of the Institute:

Development of a basic understanding of the folding of proteins into native states is essential to understanding many molecular diseases.

Proposed Course:

Intrinsically, proteins can assume enormous numbers of conformations. One plausible point of view about proteins and their folded forms is that there may not be so very many compact forms consistent with the known features of protein structure, such as our table of residue-residue interactions that essentially favors interactions among hydrophobic residues and interactions between hydrophilic residues and water.

In almost all of these macromolecular conformational problems, one of the principal problems is how to reduce the astronomically large number of conformational arrangements that appear to be possible, since all of these certainly cannot be traversed by one protein during the folding process. One way to approach this is to begin sequentially by imposing the strongest possible interactions on the protein. In this case we would favor hydrophobic pairs phe-phe, phe-met, phe-ile, met-trp, met-ile, phe-leu, ile-ile, ... in the order of their interactions energies. For any given protein, not all such favorable interactions can be physically possible, within the constraints introduced by the sequence and chain stiffness because they would rapidly lead to impossibly entangled conformations. However, you would only have to continue the process for different combinations until topologically impossible forms are encountered. Preliminary perusals of known structures indicate this to be a promising approach. In other words, we will take our table of residue-residue interactions and apply it to the sequence in order beginning with the most favorable types of interactions. First we would look at all combinations of phe-phe pairs. At each stage it is necessary to consider whether it is physically possible to form the interactions, with previous ones in place. Also we may investigate the unfolding

problem in the opposite way. The same type of approach should be applicable to the 3-dimensional RNA folding problem.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08371-03 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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 Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Ruth Nussinov	Institute of Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel	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	0.9	0.1

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.)*

Conformations of DNA 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 broad spectrum of conformations possible. Specifically there are sequence dependent variations in the vicinity of the B-form. It is interesting to consider whether or not these variations are large enough to affect recognition processes. We have investigated solvent effects on the DNA conformations and have found that some specific sequences exhibit smooth bends and some others give sharp bends.

Project Description:

Objectives:

A principal goal of molecular biology is to understand the bases of molecular and biological recognition. Complete comprehension of the important details of all interactions involved would facilitate the understanding of numerous biological processes, as well as provide a sound theoretical basis for molecular design procedures.

An ultimate goal for theory in this area remains the calculation of favored macromolecular conformations directly from their sequences. However, complexities of molecular environments make it difficult to quantitatively represent, with a single calculation method, the full range of situations in which a macromolecule may exist. 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.

One result in common to all of these studies has been to emphasize the importance of "hydrophobic" interactions or solvent effects. By this term, we mean the interactions among non-polar atoms favored by removing them from direct interactions with water. In the case of interactions between amino acids within globular proteins these "hydrophobic" interactions are significantly stronger than other types of interactions. For DNA, there is less possibility for such types of interactions, but we have proposed that they can significantly affect the path of the double helix, and can induce a range of bends, from smooth to sharp ones. For DNA operator - protein repressor interactions, we have fit experimental binding constants with models, in which these interactions can vary in strength, and we find such "hydrophobic" interactions to be one of the more significant interactions and, furthermore, to be critical in determining the specificity of binding sites.

Methods Employed:

We have been looking especially at solvent effects on the conformation of double helical DNA. If one looks at B-form double helical DNA, one of the more outstanding sequence dependent features of the major groove is the methyl group that protrudes into the major groove on each thymine. If hydrophobic interactions are so important to stabilize proteins, why shouldn't they also be important for DNA? One possible way they could arise is for these T methyls to fold together to minimize the hydrocarbon surface area for certain sequences of bases.

Major Findings:

On the basis of surface area calculations, we concluded that DNA segments containing neighboring sequences of A's or T's beyond a certain minimum number were likely to exhibit such an effect. The number of bases between such runs becomes important also, as this partly determines the direction of the bends. When the frequency of repeats corresponds to the helix repeat, around 10 bp, then a planar curve is obtained.

An example where such a planar curve would be obtained is the trypanosome kinetoplast fragment model of 47 bp sequence $ATTCCCA_5TGTCA_6TAGGCA_6TGCCA_5TCCCAA$. It can easily be folded by 10 degrees at each junction between A's to bring the methyls closer together. There are now an abundance of gel electrophoresis data to indicate that bent molecules progress several times slower through a gel than expected for their size.

Control regions of DNA are unusually rich in A's and T's; the methyls provide additional hydrophobic patches for protein binding but also might affect the conformations. Sharper bends are also possible for some other A/T sequences such as TTAA. One occurrence of this sequence is in the human disease hereditary Greek persistence of fetal hemoglobin in which the sequence TTGA is changed to TTAA. This specific change results in increased transcription of the gene. The reason for the increased efficiency is not known, but we have proposed that the conformation of the DNA at this site is bent and is consequently likely to affect the process.

Significance to Biomedical Research and the Program of the Institute:

Understanding the role of conformation variation in DNA recognition and control of transcription would permit direct understanding of a number of molecular diseases involving these processes.

Proposed Course:

A major unresolved question about DNA conformations is to what extent are conformational variations intrinsic to the sequence and to what extent are they induced by protein binding or interactions with other molecules. We have begun investigating the former question. As evidence for the intrinsic sequence effect, there are now experimental results from gel electrophoresis in which large bends are detected in isolated DNA fragments by their slower mobilities. These bends clearly depend only on the sequence.

The DNA backbone is known to be quite flexible. With this in mind, we have initiated energy calculations to determine the interdependence of the conformations of bare DNA base pairs, without backbone. We find them to be neighbor interdependent, and generally not to show extreme deviations from the standard B-form double helix. Validation requires further calculations to determine the actual limits to the range of interactions along the sequence, and also to determine the validity of ignoring the phosphate-sugar backbone associated with such forms.

It is difficult to introduce the backbone into the types of calculations described here. In conjunction with the latter, we are planning to embark on an effort to develop a computer graphics modelling program by applying "bond driving" methods developed by Dr. J. Mazur. In this scheme, a bond rotation in the center of a chain is performed, but it is implemented only in very fine steps with adjustments of surrounding atoms into energy minimized arrangements. This avoids improbable changes in the middle of a chain in which the entire arm corresponding to half of the molecule would be moved. Instead, the effects are absorbed by local adjustments of nearby atomic positions. Conceptually this process is similar to heat annealing of a glass. Such a method would have immediate applications to the present problem but also should find widespread applications in many molecular modelling problems.

Publications:

Jernigan, R. L., Sarai, A., Ting, K.-L., and Nussinov, R.: Hydrophobic interactions in the major groove can influence DNA local structure. J. Biomol. Str. Dyn., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08372-03 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

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)*

Robert Jernigan, Ph.D.,	Theoretical Physical Chemist	LTB, NCI
Akinori Sarai, Ph.D.,	Visiting Fellow	LTB, NCI

COOPERATING UNITS *(if any)*

Gene Barnett, Ph.D.,	Detail from FDA	LTB, NCI
Yoshinori Takeda, Ph.D.,	PRI, 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	0.9	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.)*

Interactions of DNA with repressor proteins, drugs, carcinogens and free radicals are being studied. Shifts in electronic structures are being calculated with standard molecular orbital methods. The aim is to determine the dependence of the interactions on the DNA sequence and conformation.

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. Protein binding gel assays have been simulated successfully.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

Several studies of molecular interactions with DNA have been initiated. These include repressor-operator binding, drug and carcinogen binding and radiation effects, as well as local conformation models of DNA.

Various detailed models for DNA-protein binding are being investigated. These are being assessed for their likeliness by comparing calculated binding energies with experimental binding constants for native operator and mutant operators. Also calculations are performed for all sites in the entire species' DNA to determine the uniqueness of binding sites.

Structure-activity relationship (SAR) studies are underway to investigate DNA adduct formation with antitumor drugs and chemical carcinogens. Preferred binding sites and sites of covalent bond formation are sought. Likewise the sequence and conformational characteristics of both radiation susceptible and protected sites are sought.

One common feature to all of our studies has been to emphasize the importance of "hydrophobic" interactions or solvent effects. By this term, we mean the interactions among non-polar atoms favored by removing them from direct interactions with water. In the case of interactions between amino acids within globular proteins these "hydrophobic" interactions are significantly stronger than other types of interactions. For DNA, there is less possibility for such types of interactions, but we have proposed that they can significantly affect the path of the double helix, and can induce a range of bends, from smooth to sharp ones. For DNA operator - protein repressor interactions, we have fit experimental binding constants with models, in which these interactions can vary in strength, and we find such "hydrophobic" interactions to be one of the more significant interactions and, furthermore, to be critical in determining the specificity of binding sites.

Methods Employed:

We have been looking in detail at molecular interaction energies between protein repressors and DNA operators. We have built models of Cro repressor-operator binding. These mostly follow the model suggested previously by Anderson, Ohlendorf, Takeda and Matthews in which an α helix is bound in the major groove. By looking at all possible interactions between base pairs and the amino acids on this helix, we have developed an interaction table for all possible interactions between this α helix and any base sequence. We have chosen to classify interactions into: charged hydrogen bonds, uncharged hydrogen bonds and hydrophobic interactions with the methyl of thymine.

Major Findings:

For the known operators, we count the interactions of each type and vary their energies to fit the binding data for the six operators of lambda phage. Results are given in Table I.

Table I. Cro Repressor Binding Ratios

Operator	Experimental	Calculated
O_R3	5.6	5.7
O_R1	1.0	0.93
O_R1	0.67	0.70
O_L2	0.48	0.51
O_L3	0.17	0.17
O_R2	0.10	0.06

This excellent fit was obtained with the following values of the energy parameters: uncharged hydrogen bond h = 0.42 kcal/mole, charged hydrogen bond h* = 0.5 kcal/mole and T methyl hydrophobic interaction m = 0.34 kcal/mole. The important features are the relative strengths of these interactions: h* is stronger than h which is stronger than m. However, all contribute to the specificity. It should be noted that these are effective interaction energies, relative to the unbound state. Thus, the hydrogen bond energies are differential ones in the sense that they represent the improvements in energies over the unbound state in which the hydrogen bonds are with solvent.

Subsequently we applied these interaction energies to calculate the binding energies between Cro protein and the entire 48,502 b.p.'s of λ phage. The distribution of binding strengths of the Cro α helix in the major groove gives a very broad non-specific binding peak and limited numbers of strong binding sites, several as strong as the weaker operators and numerous others nearly as strong.

Restriction enzyme fragments also were assayed for binding strengths to Cro. Simulations of this gel assay were performed by calculating the theoretical binding strengths of each fragment. Most of the peaks in the gel could be identified with specific fragments in the calculation. Strong binding fragments were identified in addition to the operators.

Such a method provides the possibility of theoretically specifying the region of a DNA sequence to which a protein would bind most strongly. If other binding proteins and their mode of binding were known in detail, it would be possible to construct a catalog of such binding proteins and use them to identify DNA fragments by a combination of experiments and calculations.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of electronic structure modifications in DNA from anti-cancer drugs and carcinogens may provide a better understanding of the chemistry of these processes. Understanding details of protein-DNA interactions is directly related

to many basic areas of molecular biology, including selection and design of proteins to recognize specific DNA sequences.

Proposed Course:

We hope to extend the direct experimental determination of detailed molecular interaction energies. The advent of rapid synthesis of specific DNA sequences makes possible the investigation of molecular interactions between DNA operators and protein repressors in further detail. We are planning systematically to produce synthetic operators of varying sequence, and measure their binding constants to cro Repressor. We will then develop molecular models of probable interactions and derive detailed interaction energies between molecular groups. This should yield estimates of the energies of specific types of hydrogen bonds, as well as the energies of interaction between amino acids and bases.

Publications:

Barnett, G.: Alteration of Cytosine-Guanine Interactions due to N7 Metal Cation Binding: A Structure-Activity Relationship for Cisplatin Analogues. Molec. Pharm. 29: 378-382, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08374-02 LTB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Membrane fusion: Structure, topology, and dynamics of tight and gap junctions

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

Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Section LTB, NCI

COOPERATING UNITS *(if any)*

Dr. J. Chevalier, Broussais Hosp., Paris, France; Dr. A. Aguas, Dept. Anatomy, School of Medicine, Univ. of North Carolina, Chapel Hill

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.5	.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 completed our work on the assembly of tight junctional elements upon reversal of the osmotic conditions in toad bladders implanted in Bourguet-type chambers. While reversal of osmotic conditions induces the formation of tight junctions at a topologically reversed location, the polarity of epithelial cells as evidenced by freeze-fracture morphology of apical regions, by the presence of vasopressin/cyclic AMP induced intramembrane particle (IMP) aggregates, and by the polarity of water transport, remains unaltered. We started efforts to model tight junction elements using computer assisted graphics. We completed the study of the ultrastructural alterations associated with the acrosome reaction, in particular the massive migration of integral membrane proteins and the fusion events leading to the formation of a of a complex array of tubules made from elements of both acrosomal and plasma membranes.

PHS 6040 (Rev. 1/84)

GPO 914-918

PROJECT DESCRIPTION

Objectives:

To study the structure and function of tight junctions. To induce experimentally the rapid formation of tight junctional elements and to reverse their topology in epithelia subject to osmotic reversal. To prepare computer graphics models of the structure and biogenesis of tight junctional elements. To study the ultrastructural alterations and the changes in the pattern of distribution of integral membrane proteins that mediate the acrosome reaction in sperm cells.

Methods Employed:

Conventional freeze-fracture observations of Pt/C replicas of appropriate biological specimens. Toad bladders are incubated in specified solutions, generally with reversal of the osmotic conditions. Boar sperm cells are fixed with glutaraldehyde at selected periods after induction of the acrosome reactions by calcium ionophores.

Major Findings:

We studied the effect of reversing the osmotic environment between luminal and serosal compartments of a toad urinary bladder on the polarity of assembly of tight junction strands. Toad bladders were filled with Ringer solution (220 mosm) and were immersed in distilled water at room temperature or at 37°C. Within two minutes, new tight junction strands are assembled. The new tight junctional strands unite the basal pole of epithelial cells with the apical side of basal cells. Physiological studies show that oxytocin, a synthetic analog of antidiuretic hormone, is still capable of inducing increases in water transport in epithelia which were osmotically reversed. This capacity decreases significantly for longer periods of osmotic reversal. Osmotic reversal does not alter the original polarity of epithelial cells: 1) apical tight junction belt, at the apical pole, is not displaced; 2) the freeze-fracture morphology typical of apical plasma membrane (particle-rich E faces; particle-poor P faces) is not altered; 3) oxytocin and cyclic AMP induce aggregates which are observed only at the apical plasma membrane. Massive assembly of junctional elements occurs even in epithelia pre-incubated in the presence of cycloheximide (an inhibitor of protein synthesis) or of cytoskeleton perturbers. These experiments show that the polarity of assembly of tight junction strands depends on the vectorial orientation of the osmotic environment of the epithelium.

The acrosome reaction is a secretory event of sperm cells characterized by multiple fusions between acrosomal and plasma membranes followed by exocytosis of hydrolytic enzymes that facilitate sperm penetration through the egg investments. The acrosome reaction was induced in vitro in boar spermatozoa by Ca^{2+}, in the presence of the ionophore A23187. We obtained complete views of the freeze-fractured sperm plasmalemma to study the dynamics of its large intramembrane particles (IMPs). This population of sperm-surface IMPs represents transmembrane glycoproteins with receptors for wheat germ agglutinin (Aguas and Pinto da Silva, J. Cell Biol. 97: 1356-1364). The first effect of Ca^{2+} on the sperm surface is the lateral movement of IMPs which leads to segregation of all large particles into two restricted areas of the plasma membrane (apical rim, and equatorial region). Membrane fusion begins when the redistribution of surface

IMPs is completed. WGA inhibits ionophore-induced Ca^{2+}-influx and the acrosomal reaction (Podell and Vacquier, J. Cell Biol. 99: 1598-1604); we propose, therefor that the lateral displacement of WGA-binding surface proteins may be necessary for the activation of the sperm Ca^{2+} channel. Finally, we document that fusion/fission of acrosomal and plasma membranes in acrosome-reacted sperm leads to the formation of an exquisite labyrinth of interconnecting tubules made up of hybrid membranes.

Publications:

Osmotic reversal induces assembly of tight junction strands at the basal pole of toad bladder epithelial cells but does not reverse cell polarity. J. Membr. Biol., in press.

Topology, dynamics and molecular cytochemistry of integral membrane proteins: A freeze-fracture view. J. Harris (Ed.): Electron Microscopy of Proteins. Academic Press, in press.

Freeze-fracture: Seeing and thinking biological membranes. Biomembranes and Receptor Mechanisms. E. Bertoli (Ed.): Fidia Research Series. Padova, Italy, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08375-02 LTB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Fracture-Permeation: Molecular Spacing and Structure of the Cytoplasm

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

Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Section LTB, NCI

Other Professional Personnel:

Maria L.F. Barbosa, Ph.D. Senior Staff Fellow LTB, NCI

COOPERATING UNITS *(if any)*
Dr. J. Chevalier, Dr. J. Bariety, Dr. D. Appai, Groupe de Recherches sur la Pathologie Renale et Vasculaire, (INSERM U.28), Broussais Hospital, Paris, France

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, FCRF, Frederick MD 21701

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

Fracture-permeation is a new technique developed in our laboratory to probe the compactness of the cytomatrices in glutaraldehyde-fixed cells. In fracture-permeation, macromolecules of known size are used to assess the mesh of a chemically-fixed matrix. We observed that the cross-fractured cytoplasm of freeze-fractured neutrophils was impermeable to ferritin (12 nm in diameter). We concluded the intermolecular distances within the cytoplasm of fixed neutrophils are smaller than those proposed for the intertrabecular spaces of the "microtrabecular lattice" (28-220 nm). Fracture-permeation of cardiac muscle also revealed variations in the compactness of the mitochondrial matrix and the organization of the intercristal space. In mitochondria at the condensed mode the intercristal space appeared divided into separate compartments: ferritin permeated regions adjacent to the plane of fracture but rarely diffused into spaces away from the fracture interface. In mitochondria at the orthodox configuration variations in the compactness of the matrix appear to be related to changes in metabolic activity. Work in course attempts the characterization of the extra-cellular matrix in mesangyal cells and assessment of the compactness of this matrix, as well as that of the basement membrane, by ferritin fracture-permeation and binding of cationized ferritin at different pH values.

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

PROJECT DESCRIPTION

Objectives:

1. To develop a method to investigate the existence and distribution of intermolecular spaces within the cytoplasmic and extracellular matrices of glutaraldehyde-fixed cells.

2. To study the compactness of the cytoplasmic matrix at selected stages of cellular development.

3. To study the compactness of myofilaments within the sarcomere during muscle contraction.

4. To study the chemistry of extracellular matrices in basement membranes and mesangyal matrices in rat and human glomeruli.

Methods Employed:

Cells or tissues are fixed in glutaraldehyde, impregnated with glycerol and frozen in liquid nitrogen. The cells are freeze-fractured, thawed, deglycerinated, treated with Na borohydride and immersed in concentrated solutions of globular proteins (30% w/v). From the exclusion of these probes from the cross-linked cytoplasm, we infer the existence and distribution of intermolecular spaces within the cytoplasmic matrix of glutaraldehyde-fixed cells. The cells and tissues utilized were: neutrophils from human peripheral blood, cardiac papillary muscle from rat, and kidney glomeruli both from rat and human.

Major Findings:

1. Assessment of cytoplasm compactness in human neutrophils. Neutrophils from human peripheral blood were embedded in plasma by fixation with glutaraldehyde. Freeze-fractured specimens were then immersed in concentrated solutions of ferritin (12 nm in diameter). We observed that the cross-fracture cytoplasm of mature neutrophils was impermeable to both native and cationized ferritin. Therefore, the intermolecular distances within the cytoplasm of fixed neutrophils are smaller than those proposed for the intertrabecular spaces of the "microtrabecular lattice" (29-220 nm; Guatelli et al., 1982, Biol. Cell 43: 69-80; Porter et al., 1982, 40th Annual EMSA Meeting, Washington, DC, pp. 4-7) or shown on high-voltage electron micrographs of neutrophils (Pryzwanksy et al., 1983, Eur. J. Cell Biol. 30: 112-125.

2. Fracture-permeation of cardiac muscle reveals variations in the compactness of the matrix of mitochondria and the organization of the intercristal space. Papillary muscle from the left ventricle of Sprague-Dawley rats was fixed unrestrained in 2% glutaraldehyde for 30 min at 25°C. We observed mitochondria at either orthodox or condensed configuration. Permeation of ferritin into mitochondria at the condensed mode was restricted to the intercristal space. The intercristal space appeared divided into isolated compartments: ferritin-permeated regions adjacent to the plane of fracture but

rarely diffused into spaces away from the fracture. Variations in the compactness of the mitochondrial matrix may be related to changes in metabolic activity.

Fracture-permeation and fracture-label experiments now underway test the compactness and cytochemistry of the principal cytomatrices in the glomerulus: the basement membrane and the mesangyal matrix. Cationized ferritin binds to all areas of the basement membrane, but fails to penetrate it, although it easily permeates and sparsely labels the mesangyal matrix. Permeation by native ferritin tests the existence of smaller spaces within the basement membrane matrix.

Publications:

Fracture-permeation: A technique to assess cytoplasmic compactness after glutaraldehyde fixation. J. Electr. Microsc. Techn., in press.

Ultrastructural patterns of ferritin-permeation into glutaraldehyde-fixed sarcomeres characterize stages of contraction in striated muscle. J. Electr. Miscroc. Techn., in press.

Molecular cytochemistry of freeze-fractured cells: Freeze-etching, fracture-label, fracture-permeation and label-fracture. In Miller K.R. (Ed.): Advances in Cell Biology. Greenwich, Connecticut, JAI Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08376-02 LTB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Fracture-Label: Cytochemistry of Freeze-Fractured Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
P. Pinto da Silva, Ph.D. Chief, Membrane Biology Section LMB, NCI

Other Professional Personnel

M. L. F. Barbosa, Senior Staff Fellow

COOPERATING UNITS *(if any)* Dr. F. Kan, Dept. of Anat., Univ. of Montreal, Canada; Drs. J. Chevalier, J. Bariety, D. Appal, Dept. Kidney and Vascular Path., Broussais Hosp., Paris; Drs. J. Bourquet, P. Ripoche, Biomembrane Lab., Saclay Nuclear Res. Lab., Gif-sur-Yvette, France; Drs. M. R. Torrisi, A. Pavan, Inst. Gen. Path., Sch. Med., Univ. Rome; Dr. I. Mather, Dept. Vet. Sci., Univ. of Md.

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	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.)*

Investigation of the distribution of the glycolipid GM_1 in the plasma and intracellular membranes in human neutrophils was performed by fracture-label with choleragenoid colloidal gold complexes. Both E and P faces are labeled a finding that may indicate the symmetric topology of glycolipids in these membranes and/or the association of exoplasmic GM_1 to a transmembrane protein complex. In rat liver cells we searched for the distribution of the thyroid hormone receptor sites which mediate the action of triiodothyronine (T3) in mammalian cells. Cross fractures of nuclei show that the label is associated to the heterochromatin regions and is absent in specimens pre-treated with trypsin. In rat glomeruli, isolated or in intact tissue WGA and CF fracture-label provides cytochemical access to all of the plasma and cytoplasmic membranes as well as extracellular matrices of this filtration system. Cationized ferritin labels intensely the lamina rara interna, lamina densa and lamina rara externa without the clustering proposed in previous conventional cytochemistry studies. In mammary gland tissue we continued fracture-label studies of the distribution of selected glycoproteins, in particular butyrophilin. This protein was found on the apical portion of the plasma membrane as well as over apically located vesicles as well as on the surface of intracellular lipid droplets.

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

PROJECT DESCRIPTION

Objectives:

To expand fracture-label cytochemistry through the use of affinity purified and monoclonal antibodies to detect the distribution and transmembrane topology of membrane lipids and proteins. To apply fracture-label techniques to the study of the topochemistry of the glomerulus under normal and pathologic conditions. To investigate the distribution and transmembrane topology of the glycolipid GM_1 in human neutrophils. To search for the mechanisms of traffic and the topology of proteins secreted by mammary gland epithelial cells.

Methods Employed:

In fracture-label, plasma and intracellular membranes of intact cells and tissues are labeled after freeze-fracture. Fixed tissues or cells embedded in a cross-linked matrix (e.g., BSA fixed by glutaraldehyde or cells in suspension) are treated with 30% glycerol, frozen and freeze-fractured. There are two variants of fracture-label: 1) in THIN-SECTION FRACTURE LABEL, frozen specimens are finely crushed in liquid nitrogen, thawed, and labeled. The labeled fragments are prepared for thin-section microscopy, and favorable profiles are selected from thick sections; 2) in CRITICAL-POINT DRYING FRACTURE LABEL frozen specimens are fractured under liquid nitrogen with a scalpel, thawed, labeled, critical-dried and replicated with Pt/C.

Major Findings:

The binding of cholera toxin to a specific ganglioside (GM_1) receptor provides a model for the interaction of biologically active agents with glycolipids. We developed a conjugate of choleragenoid (Subunit B of the cholera toxin molecule) and colloidal gold. In freeze-fractured neutrophils, the choleragenoid-colloidal gold complex (CTB-CG) labeled membrane halves (plasma and intracellular membranes) and the nuclear matrix. Background label was scarce or absent. Neither free choleragen nor free GM_1 were able to displace label by CTB-CG. Treatment of Z01 CB 0837-02 freeze fractured cells with trypsin abolished label on the nuclear matrix but did not alter the label of the membrane halves. Contrary to our expectations, we have observed labeling of both exoplasmic and protoplasmic halves of the plasma membrane. The unexpected labeling of the protoplasmic half of the membrane could result from (a) unspecific binding, (b) the presence of GM_1 or (c) the association of GM_1 to a transmembrane protein. Because glycolipids are believed to reside in the exoplasmic half of cell membranes, we are investigating the significance of the label observed on protoplasmic membrane halves.

We are using fracture-label to establish the ultrastructural localization of receptors for T_3 hormone in cross-fractured nuclei of liver cells. Receptors for the T_3 hormone are detected by labeling freeze-fractured liver tissue and isolated hepatocytes with a conjugate of T_3, covalently bound to bovine serum albumin (BSA), and colloidal gold (T_3-BSA-CG). The label, as observed in thin sections of cross-fractured nuclei from both liver tissue and isolated liver cells, suggested that binding sites for T_3 hormone are preferentially associated to the heterochromatin regions. As an example, in fifteen micrographs of cross-fractured nuclei from isolated hepatocytes, 74% of the label was associated to the heterochromatin, 13% to the euchromatin and the remainder, 13%, to poorly

defined regions. Evaluation of labeling was done by counting, at the fracture-plane, the number of gold particles associated to each intranuclear compartment. Colloidal gold coated with BSA (without T_3; BSA-CG) does not label the freeze-fractured cellular component. This shows that T_3, and not BSA, is responsible for the binding of T_3-BSA-CG. Trypsinization of fractured tissues or cells abolishes the original label and points to proteinaceous nature of the binding sites. Label was significantly inhibited by incubation with T_3 coupled to BSA (T_3-BSA) but was not affected by the presence of free T_3. Inhibition by T_3-BSA, in contrast to the inability of free T_3 to compete with T_3-BSA-CG, may result from a higher affinity of T_3-BSA for the nuclear receptors or from binding to sites different from the receptor.

It is our objective to determine the distribution of selected glycoproteins on intracellular and on the plasma membrane of secretory mammary cells. Mammary tissue is processed through thin section fracture label (TS-FL) and critical point drying fracture label (CPD-FL) to identify the cytoplasmic vesicles that bear the glycoprotein butyrophilin. Freeze-fractured tissues are thawed and incubated with affinity purified polyclonal antibodies against butyrophilin, followed by incubation with protein A-colloidal gold complexes. We intend to extend our study to other FGM proteins. So far we have confirmed that butyrophilin is located on the apical plasma membrane of secretory mammary cells: label was found on the E faces of the apical plasma membrane. Little or no label was seen on the P faces, an indication that butyrophilin is preferentially associated with the outer half of the apical plasma membrane. In similar samples, the nuclear envelope, endoplasmic reticulum and mitochondria remained unlabeled. Butyrophilin was also detected on apically located vesicles or on the surface of intracellular lipid droplets (the identification of this latter stucture requires further use of TS-FL). Intracellular fat droplets isolated by biochemical fractionation were shown to contain butyrophilin. If the localization of butyrophilin is confirmed, this glycoprotein, as found on milk fat globule membrane does not necessarily arrive from the apical PM as has been suggested. This observation could provide clues as to the function of butyrophilin in the formation and secretion of lipid droplets from the apical surface.

Fracture-label label and fracture permeation are used to characterize the topochemistry and Z01 CB 0837-02 compactness of the extracellular matrices of rat and human kidney glomeruli. We have shown that the basement membrane contains numerous WGA binding sites both at the lamina rara externa and interna. Work in course attempts characterization of the extra-cellular matrix in mesangyal cells and assessments of the compactness of this matrix as well as that of the basement membrane by ferritin fracture-permeation and binding of cationized ferritin at different pH values. The work can be extended to reapproach the ultrastructural pathology of kidney function, including the localization of immune complexes.

Publications:

The acrosomal membrane of boar sperm: A Golgi derived membrane poor in glycoconjugates. J. Cell. Biol. 100: 528-535, 1985.

Freeze-fracture cytochemistry of wheat germ agglutinin and concanavalin A receptors on the plasma membrane of normal, Bernard-Soulier and thrombasthenic platelets. Am. J. Pathol. 122: 292-301, 1986.

Preferential association of glycoproteins to be the euchromatin regions of cross-fractured nuclei is revealed by fracture-label. J. Cell. Biol. 102: 576-686, 1986.

A guide to fracture-label. Advanced Techniques in Biological Microscopy. I. K. Koehler, ed.). Springer Verlag, Heidelberg: 3, pp. 201-227, 1986.

Freeze-fracture cytochemistry: A poor person's guide. Bull. Electr. Microsc. Soc. Am., in press.

Molecular cytochemistry of freeze-fractured cells: Freeze-etching, fracture label, fracture-permeation and label-fracture. Advances in Cell Biology. K. R. Miller (ed.). J. A. I. Press, Greenwich, CT: 1 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08377-02 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: The Role of Myoglobin in Oxygen Transport

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

David G. Covell, Ph.D. Senior Staff Fellow LTB, NCI

COOPERATING UNITS *(if any)*: Dr. John Jacquez, Department of Physiology & Biostatistics, University of Michigan, School of Medicine

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	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 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 P_{O_2} below 10 torr does myoglobin begin to provide a measurable increase in oxygen delivery to mitochondria.

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

Project Description

Objectives:

1) To determine the role of myoglobin in facilitating oxygen delivery to tissue. 2) To establish conditions under which the nutrient supply of oxygen begins to limit the size of a tissue (or tumor) mass.

Methods Employed:

Model development is being done with the use of IMSL numerical software run on the VAX 11/780 computer system in the Laboratory of Mathematical biology.

Major Findings:

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 P_{O_2}'s below 10 torr at the surface and for diffusion lengths expected for skeletal muscle fibers.

Significance to Biomedical Research and the Program of the Institute:

A greater understanding of conditions where oxygen delivery via free or myoglobin-facilitated diffusion begins to limit the growth of tissue will be useful in the treatment of tumors.

Proposed Course:

The project is being continued with examination of models than include oxygen delivery to necrotic and non-necrotic regions of tumor tissue.

Publications:

Jacquez, J.A. and Covell, D.G.: Models in the analysis of the role of myoglobin in the diffusion of oxygen in red skeletal muscle. In, Jacquez, J.A. and Chauvet, G. (Eds): Proceedings of the Second International Colloquium on Theoretical Biology and Medicine, New York, Masson, pp. 110-120, 1986.

Covell, D.G., and Jacquez, J.A.: Does myoglobin contribute significantly to the diffusion of oxygen in red skeletal muscle? Am. J. Physiology (Resp. Physiol.), 1986, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08378-02 LTB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Label-fracture: High resolution labeling of cell surfaces

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Section LTB, NCI

Other professional personnel:

M.L.F. Barbosa Senior Staff Fellow LTB, NCI

Drs. J. Chevalier, J. Bariety, D. Appai, Dept. Renal Vasc. Path. (INSERM), Broussais Hosp., Paris; Drs. P. Ripoche, J. Bourguet, Biomembrane Lab., Saclay Nuclear Res. Lab., Gif-sur-Yvette, France; Drs. M. Torrisi, A. Pavan, Inst. Gen. Path., Sch. Med., Univ. Rome; Dr. F. Kan, Dept. Anat., Sch. Med., Univ. Montreal, Canada

COOPERATING UNITS *(if any)*

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Laboratory of Mathematical Biology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, FCRF, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.75	.75	.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 development and application of label-fracture cytochemistry was pursued. In label-fracture cells fixed or unfixed are labeled and then freeze-fractured. Label-fracture permits 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. In toad bladder epithelial cells we showed that the glycocalix over apical segments of the membrane is associated to the large intramembrane particles on the exoplasmic faces and is unrelated to the small particles that partition with protoplasmic faces. The intramembrane particle aggregates induced by vasopressin or by cyclic AMP are not labeled. In sperm cells we discovered that a particle-free zone at the base of the sperm head is heavily labeled by WGA but not by ConA. Over the sperm tail ConA labels intramembrane particles but fails to label the annulus, which is well labeled by WGA. Label-fracture of human lymphocytes during capping induced by immunoglobulins or by lectins indicates that there is no relation between the migration of surface markers and the positioning of intramembrane particles. Work under way searches for the distribution of spectrin molecules on the inner surfaces of human erythrocyte ghosts and inside-out vesicles.

PROJECT DESCRIPTION

Objectives:

To explore and develop the application of label-fracture cytochemistry to the labeling at molecular resolution of the surface of cells and organelles. Specifically; 1) to provide combined cytochemical/freeze-fracture maps of the apical membrane of toad bladder epithelial cells and to search for the topology and chemistry of vasopression induced particle aggregates thought to mediate water transport; 2) To provide detailed maps of the distribution of heterosacharides on the surface of sperm cells; 3) To investigate the mechanisms of capping in human lymphocytes, in particular to establish the presence/absence of intramembrane structural coupling mechanisms (the X-protein of Bourguignon/Singer hypothesis); 4) To study the distribution of spectrin and spectrin-like proteins on the inner surfaces of plasma membranes.

Methods Employed:

Epithelial layers of toad bladders are isolated and treated with WGA to induce aggregation of IMP's. They are these labeled by collodial gold conjugates and freeze-fractured. Sperm cells human lymphocytes and other isolated cells are labeled by lectins and colloidal gold and then freeze-fractured. Capping of human lymphocytes, induced by immunoglobulins or by lectins is followed by fluorescent light microscopy and arrested by glutaraldehyde.

Major Findings:

We discovered that pre-treatment of toad bladder epithelia leads to aggregation of the large IMP's that, in these cells, partition with E faces. WGA label-fracture intensely labels the thick glycocalix associated with the large particle domains but fails to mark the P-face aggregates induced by vasopression/cyclic AMP. A preliminary model of the structure of this membrane was proposed. In boar sperm we have established detailed, high-resolution maps of the distribution of mannose and of sialic acid/N-acetylglucosamine residues over the entire surface of the cell. Among other findings we document the existence of a IMP-free area at the base of the sperm head which is densely covered by S.A./N-A.G. but which displays no mannose residues. The distribution of WGA receptors decreases gradually over the sperm surface with the lowest density observed over the post-acrosomal area. Over the tail ConA labels the IMP-rich aggregates over the mitochondrial cord of the mid-piece but fails to label the annulus which is strongly labeled by WGA. Specialized ortogonal arrays are not labeled by ConA or by WGA. In human lymphocytes label fracture experiments study capping of surface IgG or of WGA and ConA receptors. Preliminary observations fail to indicate the co-capping of IMP's that was expected from the hypothesis of Singer/Bourguignon on the existence of an intramembrane structural mediator for capping as a transmitter/driver of the capping mechanism.

Publications:

Structural and cytochemical differentiation of membrane elements of the apical membrane of amphibian urinary bladder epithelial cells. A label fracture study. Biol. Cell 55: 181-190.

High resolution surface maps of wheat germ agglutinin and concanavalin A receptors of boar spermatozoa by "label-fracture" cytochemistry. J. Cell. Biol. (in press).

Molecular cytochemistry of freeze-fractured cells: Freeze-etching, fracture-label, fracture-permeation and label-fracture. Advances in Cell Biology. K.R. Miller (ed.). J.A.I. Press, Greenwich, CT: 1 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08379-02 LTB

PERIOD COVERED: October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Multicompartmental Analysis of Calcium Metabolism

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

David G. Covell, Ph.D. Senior Staff Fellow LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Alfred 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 ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

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

Abnormal calcium metabolism in adolescent children and postmenopausal women can have devastating consequences. The objective of this study is to elucidate the kinetics of calicum metabolism in normal children and to evaluate disease related changes in calcium metabolism in children and adults. Stable calcium isotopes were administered to children and women of childbearing age and serial samples were obtained for two to four days. Two stable isotopic tracers were used in these studies; one given orally and one given intravenously. The use of two tracers allows direct measurement of several important parameters of calcium metabolism, principally the fraction of calcium absorbed orally and the endogenous fecal excretion. Thermal ionization isotope ratio mass spectrometry was used to measure tracer enrichments in serum, urine, feces and food. The data was used to develop a multicompartmental model of calcium metabolism that better characterized calcium metabolic fluxes between regions of the body.

Most significant finding to date is that age influences the rate of calcium absorption in adult women. Women beyond the age of 45 demonstrate a significantly slower rate of absorption of dietary calcium than younger women. The affect of this finding on total body calcium metabolism has yet to be determined. We hypothesize that under conditions of increased gut motility, the slower absorption of dietary calcium may reduce total absorption, and signal the beginnings of osteoporosis.

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.

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

Project Description:

Objectives:

To develop a multicompartmental model of calcium metabolism for populations that are inaccessible to studies using radiotracers; particularly in children and women.

Methods Employed:

Model development, simulations and optimizations are being done using software available in the IMSL statistical computing package and with the SAAM computing program.

Major Findings:

Most significant finding to date is that age influences the rate of calcium absorption in adult women. Women beyond the age of 45 demonstrate a significantly slower rate of absorption of dietary calcium than younger women. The affect of this finding on total body calcium metabolism has yet to be determined. We hypothesize that under conditions of increased gut motility, the slower absorption of dietary calcium may reduce total absorption, and signal the beginnings of osteoporosis.

Significance to Biomedical Research and the Program of the Institute:

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.

Proposed Course:

The proposed course of this study is to increase the data base used to develop the multicompartmental model describing calcium kinetics. In addition to measuring calcium we will include measurements of hormones (PTH) and vitamin-D. These substances are integral participants in the control of calcium homeostasis and their roles will be included in the control portion of the compartmental model.

Publications:

Yergey, A.L., Covell, D.G., Hansen, J.W. and Vierira, N.E.: Calcium metabolism studied with stable isotopic tracers. In Turnland, J.E. and Johnston, P.E. (Eds.) Stable Isotopes in Nutrition, ACS Symposium Series 258, Washingtion D.C., Amer. Chem. Soc. Press 1985, pp.27-37.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08380-02 LTB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Molecular Structure of Animal Viruses and Cells by Computational Analysis

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

Jacob V. Maizel, Jr., Ph.D. Chief, Laboratory of Mathematical Biology, NCI

Other Professional Personnel:

Devjani Chatterjee, Ph.D.	Visiting 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
Bruce Shapiro, Ph.D.	Computer Specialist	LTB, NCI

COOPERATING UNITS *(if any)*

Dr. B. Fields, Dept. Microbiology and Medical Genetics, Harvard Medical School; Dr. D. Evans, NIBSC, London, UK; Dr. K. Currey, Dept of Neurology, Univ. of Utah

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 B

☐ (a1) Minors

☐ (a2) Interviews

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

Picornaviruses cause diseases typified by polio, colds, hepatitis, and foot-and-mouth diseases. Sequences of these viruses have been examined for relationships among them and to other known and hypothetical proteins. Secondary structures of the RNAs have been found to vary 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. Physical association between the viral E3 glycoprotein and cellular MHC proteins correlates with homology between their sequences.

Techniques of biochemistry, virology, electron microscopy and computer analysis are used to study picornaviruses and adenoviruses. Analyses of proteins and nucleic acids have been developed and implemented. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Programs are developed 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. Protein secondary structure is being predicted from amino acid sequences. New sequences are compared with computerized databases to detect relationships with known proteins.

Project Description:

Objectives:

Our objective is to describe in molecular detail the components of animal viruses and cells with the aim of pinpointing the molecules that cause genetic and non-genetic changes in transformation and infection. The animal virus systems used as models have been poliovirus, adenovirus and murine oncornaviruses. A further objective is to develop techniques to characterize the pathology of mutational and cancerous changes at the level of molecular structure.

Methods Employed:

Representatives of the genomes of picorna-, adeno-, oncorna-, or other viruses and many cellular genes have been entirely sequenced. Previous work had provided a background of biochemical knowledge that can be correlated with analytical studies of sequence properties, and extended as ideas arise from these analyses. Patterns of bases in nucleic acids and in amino acid sequences derived from them are examined with a wide variety of searching algorithms using patterns that are designed by the investigator either ad hoc from pre-conceived consideration of molecular structure or revealed from mathematical or graphic pattern-detecting techniques. General principles of protein and nucleic structure are fitted to the predictions and to biochemical and biological observations. Fast, skimming methods are used to find close relationships quickly and more exhaustive homology and structure predictions are used to detect weakly similar structures and to extract maximum detail. Derived working databases are prepared and examined by relational database techniques to produce derived knowledge bases. Strong emphasis is placed on the comparative approach. Direct structure determination is given equal emphasis in an effort to read and interpret the information of the gene sequences. Adaptation of supercomputing power to these approaches is utilized to explore new frontiers of sequence analysis and structure prediction.

Major Findings:

Picornaviruses: Secondary structure predictions of the genomes of virulent and attenuanted poliovirues types 1, 2, and 3, have shown structures near the 5' end of the genome that are conserved among strains. Bases that vary are concentrated in the unpaired secondary structure regions. Comparisons of the genomes of a number of diverse members of this virus family show a strong similarity in molecular biology and structure by a wide variety of homology searches including the graphic matrix and Goad-Kanehisa local homology methods, yet show a wide variety of biology and tissue trophisms, including neurovirulence and, in the case of hepatitis A virus, liver pathogenesis. Poliovirus type 1 RNA secondary structure was predicted by a combination of several computer programs and shown to agree broadly with electron microscope observations. This provides encouraging affirmation of the ability of structure prediction.

Collaborative studies on other sequences: Mouse beta-globin m-RNA structures were predicted by combination of the computerized folding programs, evolutionary conservation of structure, and biochemical data. The predicted structure

indicated interaction between parts of the molecule widely separated in the sequence, and is consistent with other data suggesting interaction between 5' and 3' noncoding regions in mammalian mRNAs.

Methodology: Expectations that simple pattern searches would detect constant, defined subsequences that would be unique for important features of the genome have not been realized. Yet, the information must be encoded in those sequences. Several algorithms were developed to search for patterns from more subtle viewpoints. In one approach the sequences are transformed using the Calladine-Dickerson rules for local variations in the helical structure of double-stranded DNA. A graphic matrix method was developed to display DNA secondary structural relationships allowing comparison of 2, 3 or 4 sequences at a time for any repetition of patterns. Additional computer tools have been developed to allow rapid extraction and alignment of sequences from the GenBank database using any of the several dozen feature categories included. A variety of analyses can be performed on the aligned table. Work in progress suggests that non-random distributions of simple patterns occurs at levels that are not detectable by pairwise or three-way comparisons. Sequences containing eukaryotic promotions and all available flanking seuqences within 500 bases were analyzed for "signal" oligonucleotides occurring at more or less than average expectations. Heightened occurrence of TAT/ATA rionucleotides occur at about -275 from the RNA start site as well as at the previously publicized -40 region.

Significance to Biomedical Research and the Program of the Institute:

Structural characterization of the events in viral infections has defined critical features in cellular and viral regulatory mechanisms. Sequences of many of the most important pathogenic genes are accumulating rapidly, will continue to increase. New techniques using computers will be the only way to keep up in the analysis of this data. From those analyses we can expect to develop knowledge bases that will allow biomedical researchers and medical practitioners to describe, understand and diagnose illnesses in much greater depth. This knowledge will aid in designing treatments that prevent or correct the damage caused by the pathologic genes of cancer, viruses and genetic disorders. Synthetic molecules may be designed to substitute, correct, or alter the pathogenic molecules produced by harmful genes.

Proposed Course:

We will continue to develop and apply techniques for revealing the information in nucleic acid and protein sequences. Special effort will be given to prediction of secondary structure of nucleic acids from their sequence and correlating it with biochemical data. Higher order sequence features associated with DNA structure will be examined from the point of view of interaction with other nucleic acid and protein molecules. New and refined algorithms will be implemented as we develop them or they are reported by others. If appropriate we will explore ways to use information from nucleic acid sequences to alter the replication of viruses, and modify the consequences to the cell.

Publications:

Lockard, R.E., Currey, K., Browner, M., Lawrence, C., and Maizel, J.: Secondary structure model for mouse β^{Maj} globin mRNA derived from enzymatic digestion data, comparative sequence and computer analysis. Nucl. Acid Res. In press.

Nussinov, R., Owens, J., and Maizel, J.V., Jr.: Sequence signals in eukaryotic upstream regions. Biochim. Biophys. Acta 866: 109-119, 1986.

Currey, K.M., Peterlin, B.M., Maizel, J.V., Jr.: Secondary structure of poliovirus RNA: Correlation of computer-predicted with electron microscopically observed structure. Virology 148: 33-46, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08381-03 LTB
Formerly
Z01 CB 00885-05 LTB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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 Lemkin, Ph.D. Computer Specialist IPS, LTB, NCI

Other Professional Personnel:

Lewis L. Lipkin, M.D. Chief, Image Processing Section LTB, NCI
Morton Schultz Senior Engineer IPS, LTB, NCI

COOPERATING UNITS *(if any)*

Dr. Eric Lester, Univ. of Tenn. Medical School; Dr. Peter Sondregger, Univ. Zurich Medical School.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

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.)*

GELLAB is a computer based system for the analysis of a set of 2D electrophoretic gels. It 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 the statistical methods is also a major attribute of the system. It has been applied to helping analyze a set of gels from adult human leukemias as well as axonal proteins synthesized during axonal regeneration. The objective of creating a exportable version of GELLAB (one that will run on reasonably powerful workstation microcomputers — affordable by a laboratory) is being actively pursued.

GPO 914-918

Objectives:

The imperative of technology transfer argues for the further export of the GELLAB system (software and hardware specification) so that it is within the reach of individual university laboratories. In order to do this, it is being translated to the "portable" C language available on a wide range of computer systems from micros to supercomputers.

Methods Employed:

The translation is being performed using the PSAIL translator program currently nearing completion. The major thrust of the effort this year has been in finishing the PSAIL SAIL to C translator and starting the translation of GELLAB. PSAIL has been a major effort in automatic software to perform SAIL to C conversion. As approximately 70,000 lines of SAIL code are involved in GELLAB, a completely manual conversion is unworkable. It is expected that further extensions to GELLAB will be written in SAIL rather than C. Furthermore, other programs in this laboratory and other groups using SAIL could also be easily made portable when PSAIL is finished and when PSAIL itself runs on other types of computers. Additional work was done on the PSAIL design to insure portability and language extensibility in those areas required for making GELLAB run efficiently.

Major Findings:

Additional collaborative work with Dr. Peter Sondregger in Zurich has continued both in analyzing 2D gel data bases of harvested axonal proteins and in developing new gel analysis algorithms. The collaboration with Dr. Eric Lester at University of Tennessee Medical School on human leukemias has continued and additional evaluation of the existing Leukemia data base is continuing with additional gels added this year.

Significance to Biomedical Research and the Program of the Institute:

Changes in the alternation of the protein production of a cell as a result of environmental, drug or radiation effect is a fundamental manifestation of the changes associated with malignant transformation, etc., Indeed it seems likely that differences in patterns of protein production as revealed by GELLAB may help to characterize subgroups of cells (eg.g. in leukemias) where morphology shows no difference.

Proposed Course:

The major collaboration with Dr. Peter Sondregger (U. Zurich) is continuing with additional gels of secreated (vs. harvested) axonal proteins being analyzed.

The collaboration with Dr. Lester (U. Tenn.) is continuing with additional work on enhancing the Leukemia data base and a new joint collaboration with Dr. Alvin Mauer using 2D gels to investigate oncogene transfection of human bone marrow.

Publications:

Sonderegger, P., Lemkin, P., Lipkin, L., and Nelson, P.,: Differential modulation of the expression of axonal proteins by non-neuronal cells and the peripherial and central nervous system. EMBO J. 4: 1395-1401 1985.

Lemkin, P.,: PSAIL - SAIL to C. Computer Language 2:39-45 1985.

Sondregger, P., Lemkin, P.F., Lipkin, L.E., Nelson, P.G.,: Coordinate regulation of the expression of axonal proteins by the axonal micro-environment, Dev. Biology in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08382-03 LTB
FORMERLY
Z01 CB 00886-04 LTB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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
Ruth Nussinov, Ph.D.,	Consultant	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:
2.5	2.5	0

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☐ (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.)*

Work on nucleic acid stuctures has involved a variety of collaborations and directions. Included among these are those involving global sequence homology (R. Nussinov, Tel Aviv. Univ), the study of nucleic acid molecular helical twist, torsion angle, etc., particularly in term of the energetics involved (Jernigan, Nussinov, Sarai) and a return to the problem of the analysis of structure as revealed in electron micrographs, but now as related to predicted shapes and gel migration patterns. Finally a beginning has been made on the problem of developing a nucleic acid expert sytem, which would be directed to both intellegent queries by research and a non-ad hoc structuring of the relationships among the various software/hardware complexes available at the Frederick Cancer Reserach Center.

GPO 914-918

Project Description

Major Findings:

A global sequence homology algorithm has been implemented in collaboration with Ruth Nussinov and Gaudi Landau, both from the University of Tel Aviv. This algorithm will find matches and near matches of a pattern consisting of a nucleic acid sequence against a fragment or an entire genome. The scoring scheme takes into account deletions, insertions and changes finding the positions that would require the fewest transitions. This algorithm works in order K squared n time, where K is the number of transitions allowed and n is the size of the target sequence. This and other algorithms are being studied not only from the sequence homology point of view, but also from the standpoint of utilizing such techniques for the problem of secondary structure similarity. The program is also being translated into C (partially utilizing PSAIL) to aid in portability.

The study of DNA structural variations as a function of helical twist, roll, propeller twist and torsion angle variation is still continuing. This work is being done in collaboration with Ruth Nussinov, Robert Jernigan and Akinrai Sarai. The concepts and systems previously utilized in this research are expanding, taking into account the energetics of inter-atomic reactions to see how the structural variations based on these calculations conform with some of the previously published data and to determine whether there are interesting morphologic features present at or near functional sites. The systems used previously to explore these structural variations have been expanded to include these energy calculations and some other techniques based on somewhat different fundamentals.

Associated with the problem of determining structure from sequence data is the determination of structure by analysis. Several areas are being explored here. These envolve the use of electron micrographs to measure the shapes of RNA and DNA and to relate these measurements to predicted shapes and to gel migration patterns. These same techniques should prove to be useful in measuring shape variations in DNA as a result of protein-DNA complexes.

Exploration has begun concerning the development of an Nucleic Acid expert system. This system would enable a researcher to pose queries concerning different aspects of nucleic acid and protein data bases. Rules of thumb would be incorporated into the system that would permit intelligent querying of the data base and intelligent direction of searches. The system would also contain help facilities and mechanisms to tie together the various pieces of hardware that currently exist at the Fredrick facility (e.g. Vaxes, Cray, Silcon Graphics and Apollo's). This aspect would enable a user to concentrate on the biological problem rather than on which hardware should be used and on which hardware specific algorithms reside.

Publications:

Shapiro, B.A., Nussinov, R., Lipkin, L.E. and Maizel, J.V.: A sequence analysis system encompassing rules for DNA helical distortion. Nucleic Acids Res. 14: 75-86, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08383-01 LTB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

A Mathematical Model of Anti-B-cell Monoclonal Antibody Pharmacology

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

David G. Covell, Ph.D., Senior Staff Fellow — LTB, NCI

Other Professional Personnel:

John N. Weinstein, M.D., Ph.D., Senior Investigator — LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.50	0.50	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Monoclonal antibodies or other ligands are potentially useful for the diagnosis and treatment of tumors. Their potential depends on the ability of the antibody to reach the target cell. We have developed a theoretical model for the biodistribution of B-lymphocyte specific whole monoclonal antibody and the FAB_2' and FAB' fragments following intravenous injection. The model incorporates processes for transcapillary transport of antibody via diffusive and convective pathways, the binding of antibody for B-lymphocytes, the trafficking of B-lymphocytes throughout the body, entry of antibody and B-lymphocytes into the lymphatics system and metabolic degradation of antibody.

Significant theoretical findings include the following: whole IgG a) binds rapidly to circulating B-lymphocytes located in the plasma, b) is distributed throughout the body largely in association with circulating B-lymphocytes, and c) appears to be catabolized as a consequence of B-lymphocyte cell turnover rather than catabolism of the free protein fragment. The pharmacokinetics of $F(ab')_2$ compare quite favorably with those of the whole antibody, due primarily to its comparable avidity for B-lymphocytes. In contrast, the pharmacokinetics of Fab' does not appear to be strongly influenced by association with B-lymphocytes. This observation is consistent with the lower avidity of Fab' for B-lymphocytes. Consequently, the pharmacokinetics of B-cell specific Fab' are similar to those of nonspecific Fab'.

These results provide pharmacokinetic criteria for selecting whole IgG, $F(ab')_2$ or Fab' for various in vivo applications under conditions where circulating antigens may exist.

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

Project Description

Objectives:

To investigate factors that improve the targeting of intravenously administered monoclonal antibodies.

Nonstandard Methods Employed:

(1) Computer simulation of ordinary and partial differential equations describing the theoretical model.

Major Findings:

1) The pharmacokinetics of B-lymphocyte specific whole antibody or antibody fragments are influenced by the avidity of interaction with circulating targets. Bivalent whole and $F(ab')_2$ antibody have strong avidity for B-cells and their kinetics are determined largely by those of B-lymphocyte turnover. The lower affinity Fab' fragment shows little influence of B-lymphocyte kinetics.

2) The proposed model provides a basis for analysis of antibody pharmacology in systems that include binding to tumor and circulating antigen.

Significance to Biomedical Research and the Program of the Institute:

(1) Monoclonal antibodies or other ligands are potentially useful for the diagnosis and treatment of tumors in lymph nodes. Their therapeutic and diagnostic potential depends on the ability of the antibody to reach the target cell. (2) To improve delivery of monoclonal antibodies to target cells it is important to understand how molecules are distributed throughout body tissues.

Proposed Course:

(1) Continue theoretical studies on the interaction of antibody with circulating antigens. (2) To apply these models to the design and analysis of animal experiments and human trials.

SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE
October 1, 1985 to September 30, 1986

The Laboratory of Pathology is responsible for all the diagnostic services in anatomic pathology for the Clinical Center of the NIH and has research programs in various areas of experimental pathology. A fully accredited residency program in anatomic pathology is provided for 9 residents. The Laboratory is divided into 7 sections:

A. Surgical Pathology and Postmortem Section (Dr. Ernest E. Lack, Chief)
B. Cytopathology Section (Dr. Elizabeth W. Chu, Chief)
C. Ultrastructural Pathology Section (Dr. Timothy J. Triche, Chief)
D. Biochemical Pathology Section (Dr. David A. Zopf, Chief)
E. Tumor Invasion and Metastases Section (Dr. Lance A. Liotta, Chief)
F. Hematopathology Section (Dr. Elaine S. Jaffe, Chief)
G. 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.

A. Surgical Pathology and Postmortem 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 tissue procurement nurse works closely with the staff and helps in the distribution of specimens to scientists. Clinicopathological studies in pediatric neoplasms, endocrine tumors, soft tissue sarcomas, acquired immune deficiency syndromes and breast cancer are in progress. Dr. Lack, chief of the section, has published a series of clinicopathologic case studies in germ cell tumors, renal cell carcinomas, and carotid body hyperplasia in cystic fibrosis and cyanotic heart disease.

B. Cytopathology Section

This section provides diagnostic services in cytology (both exfoliative and fine needle aspiration) and medical genetics (service and research). The chromosomal analysis includes conventional and special banding techniques (C-, G-, and R-bands) for the examination of individual chromosome pairs. During the year, approximately 6,000 cytology specimens were accessioned.

In addition to the diagnostic reports, the staff of this section collaborates closely with the clinical and surgical staff in various clinicopathologic research projects. Dr. Diane Solomon has been applying immunoperoxidase techniques to cytological materials. The cytologic diagnosis of malignant lymphoma can be extremely difficult because malignant lymphoid cells can be morphologically similar to reactive lymphoid cells. The section has developed an immunoperoxidase method employing a battery of antibodies to T and B cell markers to distinguish malignant lymphoma cells from reactive cells. Dr. Chu

continues the development of the fine needle aspiration technique. Cytogenetic studies are being conducted on an experimental model of progression from non-metastatic to metastatic tumors. Dr. Chu also serves on the staff of Tutorial on Clinical Cytology, sponsored by IAC, ASC, and AARM. She gives lectures and workshops on fine needle aspiration cytology and application of cytopathology to AIDS.

C. Ultrastructural Pathology Section

This section provides diagnostic electron microscopy services for a diverse group of Clinical Center physicians, including NCI, NIAMDD, NHLBI, NIAID, and NINCDS, as well as submitted cases from outside physicians. This past year approximately 300 cases were accessioned; over 200 were processed and diagnosed. This facility provides diagnostic training and clinical research opportunities for residents and fellows. Dr. Maria Tsokos has been doing diagnostic EM while investigating the production of matrix proteins and tissue specific proteins by neuroblastoma cells treated with differentiating agents. The results indicate that neuroblastoma shows tripartite differentiation in vitro into 3 main cell types: neuronal, Schwannian and melanocytic.

Dr. Triche, in collaboration with several other investigators at NIH and at Navy Hospital and at Fordham University, has demonstrated that a unique, previously uncharacterized tumor of childhood, peripheral neuroepithelioma (or peripheral neuroblastoma) is distinct from classic childhood neuroblastoma in all ways. Specifically, it lacks N-myc expression, unlike the majority of neuroblastomas, and instead routinely demonstrates a reciprocal (11:22) chromosomal translocation. C-myc is expressed routinely by this tumor, unlike neuroblastoma. It never produces catecholamines (unlike 95% of childhood neuroblastomas) but instead contains purely cholinergic neurotransmitter enzymes (choline acetyl transferase). No other oncogene has so far been identified. Clinical studies based on these studies are underway, and early results (11 months median follow-up) indicate a prognosis inferior to Ewing's sarcoma (with which it is often confused) and vastly superior to disseminated neuroblastoma (which was the historical diagnosis).

Another area of active investigation by Dr. Tsokos and Dr. Triche is childhood rhabdomyosarcoma. Tumor material has been characterized by immunocytochemistry, tissue culture, and monoclonal antibody studies, as well as conventional histology. Based on information from these studies, two basic types of rhabdomyosarcoma have been identified. A retrospective analysis of 159 cases from NCI and St. Jude Children's Research Hospital has shown strong correlation between these criteria and prognosis, unlike any other criteria previously identified. As a result, an international review panel has been convened by Dr. Triche, Dr. Hal Maurer (Chairman of the Intergroup Rhabdomyosarcoma Study, or IRS), Dr. Giulio D'Angio (President of SIOP, the International Society of Pediatric Oncology), and Dr. William Newton (Pathologist-in-chief in the IRS). This panel will propose morphologic criteria for categorization of childhood rhabdomyosarcoma into favorable and unfavorable histology, substantiated by statistical analysis, and to be employed by all cooperative studies in the western world. This will be the first time such uniformity of criteria for treatment purposes will have been achieved in pediatric oncology.

Dr. Triche's group has determined that the type of matrix molecules produced by pediatric "round cell" tumors can be a clue to their cell of origin. He has characterized a previously undescribed high molecular weight matrix protein. The function and composition of this protein is unknown. Dr. Tsokos has found that neurone-specific enolase is a reliable marker for primitive childhood tumors of neural origin. This marker should be of clinical application by the pathologist in the differential diagnosis of neuroblastoma versus other solid tumors of childhood.

D. Biochemical Pathology Section

The Biochemical Pathology Section is carrying on research on immunochemistry of complex carbohydrates. Current approaches include 1) determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures by gas chromatography/mass spectrometry (GC/MS), 2) development of hybridoma antibodies against oligosaccharide haptens, and 3) immunochemical studies of cell surface glycoproteins and 4) nucleotide sequencing of hybridoma antibodies of Vh-GAC family.

Carbohydrate chains released by trifluoroacetolysis of whole tissues, tissue fractions, or cells grown in culture, are easily recovered in nearly quantitative yield and reconstituted to their native form. Analysis of the majority of oligosaccharides containing six or fewer monosaccharide units is performed by combined gas chromatography and mass spectrometry of permethylated, N-trifluoroacetylated oligosaccharide derivatives. Analysis for certain specific oligosaccharides is carried out by radioimmunoassay using antibodies produced against purified oligosaccharides coupled to polypeptide carriers. It is anticipated that the repertoire of oligosaccharide chains produced by cells or tissues will reflect states of cellular differentiation and reveal potential cell surface markers.

Membrane glycoproteins behave as either carbohydrate or peptide antigens and occasionally express antigens that combine specific structural elements from both sugar and peptide moieties. Immune responsiveness to cell surface glycoproteins has not been studied systematically. They are characterizing the fine specificities of autoantibodies against glycoproteins of human erythrocytes from patients with altered immunologic states. In addition, they are preparing hybridomas that secrete monoclonal antibodies against various portions of the carbohydrate and peptide moieties of human glycophorin A, the major sialoglycoprotein of human erythrocytes.

The mouse immune response to streptococcal group A carbohydrate (GAC) utilizes many different gene segments from a diverse array of precursor Vh genes. The gene family number is estimated at 10 and the diversity of response in normal mice at over 200 different antibodies. The response is clonally restricted in each mouse immunized, but each mouse appears to express a different clonally homogeneous antibody in its serum in spite of genetically common background. The common denominator of similarity in these antibodies is their usage of highly homologous Vh gene segments, and identical Dh, and Jh segments. The serum response to GAC is entirely deficient in CBA/N mice which makes this an ideal system in which to investigate possible constraints on gene segment utilization.

E. Tumor Invasion and Metastases Section

The Section of Tumor Invasion and Metastases is studying the biochemical and molecular genetic mechanisms which play a role in tumor cell invasion and metastases formation. This group has identified three biochemical factors: a) specific new types of proteases, b) a new type of matrix receptor and c) a new type of autocrine motility factor (AMF). All factors are quantitatively enhanced in actively invading tumor cells. Antibodies against the protease react with actively invading breast carcinoma cells in tissue sections, and are therefore of diagnostic usefulness. Blocking the proteases inhibits tumor cell invasion in vitro. Blocking the matrix receptor abolishes experimental metastases in mice. This receptor can also be readily measured in human breast carcinoma tissue. Attempts to correlate receptor content with clinical stage of disease have demonstrated a higher content of exposed laminin receptors in breast cancer cases with 2 or more lymph nodes positive for metastases compared with cases with no metastases. Clinical trials are now being set up to study the localization of metastases by labeled laminin fragments.

In order to further study the interaction of tumor cells with laminin, a glycoprotein of basement membranes, Dr. Liotta's group identified the domain of the laminin molecule which attached to the tumor cell. In addition, a specific cell membrane receptor for laminin was identified by Dr. Liotta's group for the first time. By rotary shadowing electron microscopy, performed by Ms. Margulies, laminin has the configuration of a cross with 3 short arms and one long arm. The cell binding site resides near the intersection of the short arms. The type IV collagen binding domain of laminin is associated with the globular end regions of the short arms but not the long arm. The long arm contains a major binding site for basement membrane proteoglycan. The laminin receptor has a Kd of 2×10^{-9}, 10-100,000 receptors per cell. The receptor is present on isolated cell membranes and is destroyed by trypsin. The receptor was isolated from cell membranes by detergent treatment, HPLC, nitrocellulose blotting and/or laminin affinity chromatography. The laminin receptor molecular weight is 67,000. A fragment of the laminin molecule which binds to the receptor and blocks attachment will inhibit metastases formation in animal models. Laminin receptors can be readily measured in surgical specimens of human breast cancer tissue. Monoclonal antibodies have been prepared to the human laminin receptor.

The interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. To better understand the protein components that make up the extracellular matrix and how they are regulated, this section has undertaken to construct, isolate, and characterize molecular clones of laminin receptor and of several different collagens. Using a monoclonal antibody directed against the laminin-binding domain of laminin receptor, we screened a human endothelial cell cDNA λgt11 library. Six plaques showed an intense reaction with the antilaminin receptor monoclonal but showed no reactivity toward a variety of control antibodies. The sizes of the cDNA inserts of the six clones ranged from 450 to 950 base pairs. Restriction enzyme mapping and Southern hybridization identified a 450 base pair fragment which was identical in each cDNA clone, suggesting that this fragment may represent the laminin-binding domain of laminin receptor. Several cyanogen

bromide fragments within this domain were predicted based on cDNA sequencing. One of these, a unique octapeptide, matched completely with a cyanogen bromide fragment of purified laminin receptor, thus definitively identifying the six cDNA clones as encoding part of the human laminin receptor. The laminin receptor cDNA clone recognizes a 1700 base mRNA, which is sufficient in length to code for a protein with the expected size of laminin receptor. In addition, cDNA clones coding for mouse type IV collagen and genomic clones coding for human type II collagen have been characterized.

Type IV collagenase is an important basement membrane degrading metalloproteinase that malignant cells produce and secrete to facilitate their traversation through blood vessel walls as well as during transition from in situ to invasive carcinoma. Type IV collagenase was found to be markedly agumented following ras oncogene transfection. Human type IV collagenase was isolated from culture supernatants of A2058 metastatic melanoma cells after four steps of purification. The collagenase has a molecular weight of 70 KDa and produced a typical cleavage pattern of type IV collagen by SDS gel electrophoresis. A second polypeptide was eluted off the collagen IV affinity column which has a molecular weight of 100 KDA. This new collagen IV binding protein has no collagenolytic activity and does not possess any characteristics of known matrix proteins.

This section is studying biochemical events characteristic of malignant tumor cells, which must be highly motile while invading tissue and metastasizing to distant sites. They have 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 $\sim$ 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. 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.

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 ability of ras^H genes to induce metastatic potential as well as transformation and tumorigenicity was demonstrated in several cell types. NIH-3T3 cells transformed with either the DNA of the Harvey Sarcoma Virus or with the cloned T24 human ras^H oncogene resulted in cells which formed lung metastases. Early passage diploid rat embryo fibroblasts transformed by ras^H were also metastatic as were early passage Chinese hamster lung fibroblasts transformed by ras^H. Thus, the ras^H oncogenes can induce metastatic behavior even in diploid fibroblasts. Karyotypic abnormalities were not required for metastases induction in this system.

However, transformation itself or the ability to grow as a tumor was shown to be insufficient to result in metastasis. The normal cellular counterpart of the ras^H oncogene can also transform NIH-3T3 cells if an LTR is placed upstream from the c-ras^H gene to increase the levels of the normal P21. The NIH-3T3 cells transformed by this construction, while highly tumorigenic, do not metastasize.

Ras^H did not induce metastatic potential in all recipient cell lines tested. For example, C127 cells, a murine epithelioid line which were transformed by ras^H, did not metastasize although morphologically transformed and highly tumorigenic. The induction of metastatic potential in those cells is being attempted in gene transfer experiments.

F. Hematopathology Section

The Hematopathology Section conducts a major program in diagnostic and experimental hematopathology. Drs. Jaffe and Cossman are board-certified pathologists who take responsibility for diagnostic hematopathology for patients admitted to the Clinical Center. While most of this material pertains to patients with malignant lymphoma admitted to the clinical services of the National Cancer Institute, collaborations are also conducted with NIAID and NIAMDD for patients with reactive lymphoproliferative lesions. Drs. Jaffe and Cossman also receive several hundred cases submitted for diagnostic consultation from pathologists in the regional medical community as well as throughout the United States and other parts of the world.

The Hematopathology Section continues its active research program on the immunological characterization of malignant lymphomas. All patients with newly diagnosed lymphomas or recurrences are studied for phenotypic and functional markers. This information is utilized to study the relationship of malignant lymphomas to the normal immune system, to develop improved classifications of disease, and to distinguish new clinicopathologic entities. This information is also being used as a basis for immunotherapy in collaboration with the Medicine Branch, DCT, and the Biological Response Modifier Program in Frederick,

Maryland, NCI. Based on the studies of the Hematopathology Section, a classification of post-thymic T-cell malignancies has been proposed. This classification delineates at least five major clinicopathologic entities with differing clinical presentations and prognoses.

A functional study of follicular non-Hodgkin's lymphomas showed that the neoplastic cells in virtually all cases could be induced to secrete monoclonal immunoglobulin. However, induction of immunoglobulin secretion required the addition of allogeneic helper T lymphocytes. T cells derived from involved lymph nodes were not capable of mediating such help, and in fact, appeared to exert a suppressive effect for immunoglobulin secretion in in vitro culture. Another study demonstrated that in some patients with follicular lymphoma, (a monoclonal B-cell tumor), mature T cells were numerically predominant. However, morphologically and phenotypically the infiltrating T cells appeared to be non-neoplastic. The infiltrating T cells may represent a positive host immune response, since the patients continued to pursue an indolent clinical course, even with apparent histologic progression. Both of these studies suggest that the neoplastic cells of follicular lymphoma are susceptible to host immunoregulation.

Dr. Cossman initiated a new research program over the past year regarding the molecular basis of the diagnosis of human lymphoproliferative disease. This work has already produced a number of major findings. The T- and B-cell lineage of diffuse, aggressive lymphomas as determined by phenotypic studies can be confirmed by detection of appropriately rearranged T-cell receptor or immunoglobulin genes. Moreover, in some patients' tissues which lack evidence of malignant lymphoma by conventional histology or immunologic studies, malignant lymphoma can be identified by these techniques. Molecular genetic analysis has also identified that a subclass of diffuse, aggressive lymphomas contain the t14,18 chromosomal translocation characteristic of follicular lymphoma. This observation suggests that many of these high grade B-cell lymphomas may originate as follicular lymphomas both clinically and at the molecular level.

Precursor T-cell neoplasms were shown to occupy sequential differentiation compartments during early T-cell development as shown by the coordinate expression of surface membrane molecules and T-cell receptor rearrangements. Monoclonality and T-cell lineage were also demonstrated by rearrangement of the T-cell receptor gene in Tγ lymphoproliferative disorder, a disorder in which a neoplastic basis had been questioned. Angio-immunoblastic lymphadenopathy, currently considered an atypical reactive process with a high incidence of progression to malignant lymphoma, in all cases was shown to contain monoclonal rearrangements of either immunoglobulin genes, T-cell receptor genes, or both simultaneously.

Although in most patients with malignant lymphoma, sequential studies show clonal fidelity by these analyses, in follicular B-cell lymphomas there is a high frequency (35%) of clonal evolution. Such clonal evolution allows for the escape of the malignant cells from identity by anti-idiotypic antibody. In one case, alteration of idiotype was likely attributable to somatic mutation of a V region gene. Other mechanisms of clonal evolution may include new variable gene rearrangements and heavy chain constant region isotype switching. Deduced

geneologies of the related cloned argue for an immature follicular lymphoma progenitor cell in which immunoglobulin genes remain in a germline configuration. Although new rearrangements continue to occur in the productive allele, there is conservation of the immunoglobulin allele involved in the t14,18 translocation characteristic of follicular lymphomas. These observations have important significance for treatment strategies for the follicular lymphomas which, although clinically low-grade, have been refractory to cure with current regimens. Moreover, the capacity of follicular lymphomas to frequently alter their surface membrane idiotype may allow them to escape detection by the host immune response and may account for the clinically observed waxing and waning clinical course of disease.

Dr. Neckers participates in the clinical work of the Hematopathology Section by supervising the cell sorter used in clinical phenotypic studies. Dr. Neckers also conducts an active research program on the regulation of cell growth by transferrin receptors. Major findings include the fact that normal cells regulate transferrin receptor appearance by carefully controlled tissue-specific growth factors and their receptors. However, in malignant cells this regulation is lost and transferrin receptor expression becomes constitutive. Nevertheless, both growth factor dependent and constitutive transferrin receptor expression is calcium dependent while transcription of the IL2 receptor gene and genes for C-myc and C-myb are not calcium dependent. Transferrin receptor redistribution and its associated hyperphosphorylation can be dissociated by a blockade of calcium channels. Hyperphosphyorlation is not calcium dependent but redistribution is and may reduce surface receptor levels by 60% without affecting proliferation. Cyclic AMP terminates transferrin receptor gene transcription in normal and some malignant cells and is correlated with a G1 arrest.

G. Office of the Chief

Dr. David Levens has developed a novel method for identifying sequence-specific DNA binding proteins. A well-characterized protein-DNA interaction is used to isolate from crude cellular extracts or fractions thereof proteins which bind to specific DNA sequences; the method is based solely on this binding property of the proteins. The DNA sequence of interest, cloned adjacent to the lac operator DNA segment is incubated with a lac repressor-beta-galactosidase fusion protein which retains full operator and inducer binding properties. The DNA fragment bound to the lac repressor-beta-galactosidase fusion protein is precipitated by the addition of affinity-purified anti-beta-galactosidase immobilized on beads. This forms an affinity matrix for any proteins which might interact specifically with the DNA sequence cloned adjacent to the lac operator. When incubated with cellular extracts in the presence of excess competitor DNA, any protein(s) which specifically binds to the cloned DNA sequence of interest can be cleanly precipitated. When isopropyl-β-D-thiogalactopyranoside is added, the lac repressor releases the bound DNA, and thus the protein-DNA complex consisting of the specific restriction fragment and any specific binding protein(s) is released, permitting the identification of the protein by standard biochemical techniques. He demonstrated the utility of this method with the lambda repressor, another well-characterized DNA-binding protein, as a model. In addition, with crude preparations of the yeast mitochondrial RNA polymerase, he identified a 70,000-molecular-weight peptide

which binds specifically to the promoter region of the yeast mitochondrial 14S rRNA gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00853-33 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Surgical Pathology

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

PI: E.E. Lack Chief, Surgical Pathology & Postmortem Section LP NCI

OTHER: (see next page)

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology & Postmortem Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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 and Postmortem Section, together with the Cytopathology Section, Ultrastructural Pathology Section and Hematopathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all institutes in those investigations which involve the use and study of human pathological material. A new frozen section and surgical pathology processing area was constructed adjacent to the new operating rooms and has been in use since April, 1983. This new facility has greatly enhanced processing of specimens and communication of diagnostic findings with attending physicians. It is equipped with intercom and television viewing screens in each operating room to facilitate communication.

The staff is actively engaged in a variety of projects involving clinicopathological correlation and pathologic characterization of diseases studied at the Clinical Center. Up-to-date immunocytochemical techniques have been applied to the study of tumors and other non-neoplastic diseases. The use of immunohistochemical staining has greatly facilitated more precise diagnosis in selected cases and with the increasing number of monoclonal antibodies available this technique should have even greater value in diagnostic and research pathology. A major renovation of the histology laboratory is in the planning stage. This will include space allocated for performance of special stains and immunocytochemistry. Renovation of the autopsy facility is essentially complete and its use has greatly enhanced the service.

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

Other Professional Personnel:

*T.J. O'Leary	Acting Chief, Autopsy Service	LP NCI
M.J. Merino	Asst. Chief, Surg. Path. & Postmortem Section	LP NCI
+P. Cohen	Medical Staff Fellow	LP NCI
+M Sadofsky	Staff Fellow	LP NCI
+A.C. Larner	Staff Fellow	LP NCI
+S.B. Vande Pol	Staff Fellow	LP NCI
+M.R. Lieber	Staff Fellow	LP NCI
+S.-H. Lo	Staff Fellow	LP NCI
+S.M. Mackem	Staff Fellow	LP NCI
+M.A. Thompson	Staff Fellow	LP NCI
+E.H. Radany	Staff Fellow	LP NCI
G.J. Bryant	Expert	LP NCI
C.R. Simrell	Jr. Staff Pathologist	LP NCI
D. Solomon	Pathologist	LP NCI
E.S. Jaffe	Chief, Hematopathology Section	LP NCI
T.J. Triche	Chief, Ultrastructural Pathology Section	LP NCI
J. Cossman	Senior Investigator	LP NCI
C. Restrepo	Expert	LP NCI
E. Lipford	Expert	LP NCI
*L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
*D.A. Katz	Neuropathologist	OCD NINCDS
*K.J. Stromberg	Consulting Pathologist	LVC NCI
*W.C. Roberts	Chief	IRPA NHLBI
*M. Valsamis	Visiting Consultant - Bird Coler Hospital, New York, NY	
*J. Stern	Consultant in Dermatopathology	

Project Description

Objectives:

The objectives of the Surgical Pathology and Postmortem Section are: (1) to provide diagnostic services in pathologic anatomy to the clinical research projects conducted at NIH; (2) to carry out independent research; (3) to provide a residency program in anatomic pathology; and (4) to collaborate with investigators in research involving the use and study of human materials.

The service functions of the section during the past year included:

(a) 153 autopsy examinations. The residents perform nearly all of the postmortem dissections under supervision of various staff members. A conference is held weekly by the staff and residents where gross organs are reviewed and clinical pathologic correlations are made. The residents review the microscopic slides from each autopsy they perform with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the section participates.

+These physicians are full-time Residents in the Laboratory of Pathology.
*These Associate Pathologists spend part time in the activities of the Surgical Pathology and Postmortem Section.

(b) Approximately 5000 specimens were accessioned in the Surgical Pathology Section. The in-house surgical pathology specimens are initially examined and prosected by a resident under supervision of attending staff. Associate pathologists from other laboratories or sections are frequently consulted about diagnostic problems in surgical pathology and/or assume responsibility for handling certain tissue removed for specific research projects. Approximately 600 consults were rendered.

(c) Histological preparation and special procedures: The functions of the histology laboratory are carried out under the direction of Mrs. Ruby Howard. More than 53,000 slides were prepared. Of these, about 4/5 were routine H&E stained and 1/5 were specially stained. The laboratory is very active in specialized immunocytochemistry.

(d) Photographic services of the department: A photographic record is made of much of the unusual pathological material which is handled and studied in the department. The photography is done by the staff and residents with the technical assistance of Mr. J. Banks who also maintains the equipment and helps with much of the photographic processing. Gross photographs and photomicrographs of the pathologic material are provided to the clinical staff on request, and are used extensively for conferences and various seminars. Many are prepared for publication. Over 325 black and white negatives and over 640 black and white prints have been prepared from autopsy and surgical pathology material. More than 50 35 mm rolls have been prepared from gross specimens seen on the surgical and autopsy service.

A large proportion of the photomicrography is done under the direction of Mr. Ralph Isenburg, who provides professional assistance and facilities for the entire staff of the Laboratory of Pathology.

Conferences. The staff takes part in the following inter-departmental conferences:

Pediatric Oncology Tumor Board (weekly)
Sarcoma Staging Conference (weekly)
Surgical Morbidity and Mortality Conference (monthly)
Medicine-Radiation Conference (weekly)
Surgical Pathology Conference (weekly)
Lymphoma Staging Conference (weekly)

In addition, the staff participates in clinicopathological conferences discussing specific patients.

Data retrieval system. In conjunction with DCRT, a program has been created for storing and retrieving the surgical pathology and autopsy material and to automatically encode all the diagnoses. The language used is a modification of SNOP and a dictionary is being constantly updated in order to accommodate a maximum of currently used terms.

Proposed Course of Research:

(a) Continue to provide the services described. (b) Increase the interaction with the clinical branches in the design and evaluation of protocols. (c) Improve the opportunities for the resident staff to participate in teaching, conferences, and seminars, and provide elective periods to be spent accomplishing research projects with the senior staff. (d) Implement data retrieval programs.

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Vande Pol, S. and Holland, J.: Mutations in the glycoprotein of vesicular stomatitis virus associated with resistance to natural killer cells and tumorgenicity of persistently infected cells in nude mice. J. Gen. Virol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09145-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Neuropathology

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

PI: D.A. Katz Neuropathologist OCD NINCDS

COOPERATING UNITS *(if any)*

Surgical Pathology and Postmortem Section, LP, NCI
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.)*

Subspecialty expertise in diagnostic neuropathology is provided to the Laboratory of Pathology, NCI, and to all other institutes, via the Office of the Clinical Director, NINCDS. The neuropathology service is integrated with the Surgical Pathology and Postmortem Section, and also, more recently, with the Ultrastructural Pathology Section. The service also functions in a collaborative manner to provide neuropathological expertise in a variety of clinicopathologic investigations.

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

Project Description

Objectives:

The objectives of the neuropathology service parallel those of the Surgical Pathology and Postmortem Service: (1) to provide a specialized diagnostic service for neurosurgical and autopsy material from NIH patients; (2) to use this material to carry out clinicopathologic studies of primary neurologic disease and neurologic complications of systemic disease, and to (3) teach resident trainees in anatomic pathology the fundamentals of neuropathology; (4) to assist, in collaborative fashion, basic investigators who desire to study human nervous tissue.

Autopsy: As in previous years, the brain was examined in approximately 100 cases, and at least 25% of these manifested significant primary or secondary neurologic findings. Neuropathologic consultation is available at the time of autopsy, as needed, for special handling of the brain and/or spinal cord. Detailed and standardized gross examination, description and photography are carried out with the pathology residents at weekly brain cutting sessions. The microscopic slides of all brains and spinal cords are reviewed by the neuropathologist, and the findings integrated into the autopsy report. Presentations of pertinent findings at gross autopsy conference and other clinical conferences are performed by the resident in consultation with the neuropathologist.

Surgicals: Approximately 175 neurosurgical specimens are examined yearly, including both submitted and in-house cases. Approximately 35 intra-operative frozen section consultations are provided yearly. Current in-house case material includes pituitary adenomas and stereotactic biopsies for deep-seated tumors and AIDS-related lesions.

Conferences: The case material described above is also utilized for neurologic conferences, including presentations at NINCDS Grand Rounds.

Proposed Course of Research:

Incorporation of the neuropathology material into the data retrieval system will allow for improved resident teaching, with creation of a separate teaching file, and will facilitate restrospective case studies.

Detailed protocols for the safe handling of tissue from patients with known or suspected Kreutzfeldt-Jakob disease, and for utilization of brain biopsy, are in progress.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00852-33 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Exfoliative Cytology Applied to Human Diagnostic Problems and Research Problems

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

PI:	E.W. Chu	Chief, Cytopathology Section	LP NCI
OTHER:	D. Solomon	Pathologist	LP NCI
	Y. Ye	Visiting Fellow	LP NCI
	T.A. Wood	Biologist	LP NCI
	L. Galito	Biologist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	4	4

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

A

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

The Cytopathology Section provides complete diagnostic service in exfoliative cytology, medical cytogenetics, and fine needle aspiration cytology. The section also routinely applies immunocytochemistry techniques to affirm and/or enhance cytological diagnostic efficacy. In addition, the section collaborates in various clinical research projects utilizing special techniques including special staining, tissue culture techniques, as well as investigating chromosomal and/or somatic cell hybridization techniques in mapping genes.

PHS 6040 (Rev. 1/84)

GPO 914-918

Other Professional Personnel:			
	A.M. Wilder	Biologist	LP, NCI
	K. Nakahara	Biologist	LP, NCI
	E. Sanders	Bio. Lab. Tech.	LP, NCI

Project Description

Objectives:

1. To provide the staff of the Clinical Center with an accurate and complete exfoliative and aspiration cytology service as well as diagnostic cytogenetics (karyotyping, high resolution analysis, and in situ hybridization).
2. To collaborate in various clinical research projects evaluating cancer therapy, the hormonal status of the cancer patients, the course and natural history of the cancer lesions and the anatomical and physiological changes in the human body associated with various pathologic conditions.

The specific objectives include:

A) Cytology

1. Continued improvement in cytologic techniques in establishing definitive diagnosis.
2. Improvement in fine needle aspiration cytologic technique.
3. Establishing cytologic characteristics of cell types seen only in fine needle aspirations.
4. Setting up special stain techniques which may be used for general cytology or special research problems.
5. To use immunoperoxidase technique on cytological material to enhance the establishment of definitive diagnoses.
6. Maturation index in precocious children.

B) Diagnostic Cytogenetics

1. Improvement in short-term culture system to assure of successful harvesting of in vitro cells.
2. Improvement in staining techniques.
3. Improvement in special chromosomal analytical techniques such as banding by various methods, including high resolution chromosomal analysis.
4. Gene mapping by chromosome in situ and somatic cell hybridization.

C) Research Cytogenetics

1. Chromosomal characterization of oncogene transformed rat cell lines.
2. Cytogenetic studies of metastases (animals).
3. Chromosomal in situ hybridization of ras-transformed cells.

Methods Employed:

1. Special procedures in addition to the standard Papanicolaou stain are used:

   A. Millipore filtration of and cytocentrifuge all fluids
   B. Special stains
      1. Feulgan reaction for sex chromatin bodies
      2. Acid fast, Pneumocystis, Methenamine silver, Brown Brenn for various organisms and fungi
      3. PAS, Wright Giemsa, iron stain, melanin stain, congo-red, oil-red-O, mucicarmine for various specific reactions

2. Short-term cultures of peripheral blood, bone marrow cells and tumor cells found in body fluids are the standard diagnostic methods.

In addition, special techniques involving trypsin digestion followed by special stains such as G-banding, Q-banding, and C-banding, as well as fluorescence staining whenever indicated. Also, high resolution chromosomal analysis and autoradiography are performed whenever needed.

Major Findings:

1. Providing prompt services and early diagnosis in cytological materials.
2. Providing cytologic evaluation and estimated ranges of either relapse or remission in the continual management of meningeal leukemia.
3. Providing cytological evaluation of therapeutic effects on cells seen in urinary specimens, vaginal-cervical smears, sputum and effusions.
4. Providing chromosomal analysis for clinical studies as well as in the establishment of definitive malignancies.
5. By using chromosomal hybridization techniques, certain oncogene and immunoglobulin genes can be localized.
6. Providing cytologic methods to research projects; specifically, fine needle aspiration in thyroid nodules.
7. Immunohistochemical technique often offers means to differentiate reactive cells from cancer cells in body fluids by using suitable antisera.

Significance to Biomedical Research and the Program of the Institute:

1. The diagnostic value of exfoliative cytology in the clinical management of various disease states is an established fact.
2. Ability to discern specific therapeutic effects is crucial in patient (therapy) management.
3. Aspiration cytologic techniques are useful in establishing diagnosis on metastatic diseases and/or recurrent malignancies. In such instances, the fine needle aspiration technique eliminates the more involved incisional biopsy and therefore is time saving and more economical to the clinicians as well as to the patients.
4. Immunocytochemical techniques may offer early resolutions to equivocal cases.
5. Immunoperoxidase technique can support the clinical diagnosis of malignancy with specific monoclonal antibodies.

6. Chromosomal analysis is a way of establishing definitive diagnoses in selected patients with endocrine disorders, congenital defects and also in some malignancies.
7. Cytogenetic studies abet and confirm the ongoing research work on tumor progressions.
8. Chromosomal, high resolution, and/or somatic hybridization techniques will eventually open the way for extensive experimental tests involving genes.

Proposed Course of Research:

1. Continue to provide a complete cytodiagnostic service for the entire staff of the Clinical Center in various research projects.
2. Continual improvement and development of established as well as new techniques in the cytological field.
3. Continual work with gene mapping by hybridization technique.
4. Develop and improve prophase banding technique.
5. Intensive pursuit of immunocytochemical techniques to improve and abet diagnostic cytology.

Activities:

1. Presented cytologic findings in AIDS patients. 33rd Annual Meeting of ASC, November 1985.
2. Presented cytologic findings in AIDS patients. Washington Society of Pathologists, October 17, 1985.
3. Seminar on AIDS. Metropolitan Washington Society of Cytology, February 1986.
4. Program Chairman, Annual Scientific Meeting Chinese Medical and Health Assoc., September 1986.
5. President, Chinese Medical and Health Association, 1986.

Coming Events:

1. To lead 25 M.D.'s to medical institutes in Taiwan in November 1986 and to participate (as speaker and discussant) at the Taiwan University Medical College Annual Scientific Meeting, November 1986.
2. To give a course on clinical cytology in China (Beijing and Shanghai).

Publications:

Ramacciotti, C.E., Pretorius, H.T., Chu, E.W., Barsky, S.H., Brennan, M.F., and Robbins, J.: Diagnostic accuracy and utility of aspiration biopsy of thyroid nodules. Ann. Intern. Med. 144: 1169-1173, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00897-03 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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	Pathologist	LP NCI
OTHER:	E.S. Jaffe	Chief, Hematopathology Section	LP NCI
	E.W. Chu	Chief, Cytopathology Section	LP NCI
	Y. Yee	Visiting Fellow	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:
1	1	0

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To improve the accuracy of the cytological diagnosis of non-Hodgkin's lymphoma using immunocytochemistry.

Methods Employed:

In addition to routine cytological preparation using standard Millipore filtering techniques and cytospin preparations, aliquots of each specimen are studied using the avidin biotin immunoperoxidase technique and antibodies to B and T cell markers.

Major Findings:

We have investigated 128 specimens, including 86 pleural effusions, 13 ascites, 4 cerebrospinal fluids, and 21 fine needle aspiration specimens. We have found 73 cases to be positive for lymphoma and 52 to be reactive in nature. Of the 73 positive cases, 54 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 11 cases on the basis of aberrant marker phenotype or TdT positivity.

Significance to Biomedical Research and the Program of the Institute:

The cytopathologist can play an important role in staging and diagnosis of recurrences of non-Hodgkin's lymphoma by the examination of fluids and fine needle aspiration biopsies. In most low-grade lymphomas, however, the cytological features of the malignant lymphoid cells are indistinguishable from those of non-neoplastic lymphocytes. We have developed a technique that provides a definitive cytological diagnosis of malignant lymphoma providing a sound basis for staging, evaluation of recurrence, and therapy.

The combination of immunocytochemistry and fine needle aspiration biopsy cytology is a powerful one and given the appropriate cell markers, provides unparalleled opportunity for accurate pathologic diagnosis with the minimum of patient morbidity and expense.

Proposed Course of Research:

We plan to continue our investigation of lymphoid markers on cytological specimens and to extend this approach to the diagnosis of non-lymphoid tumors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09128-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Cytologic Diagnosis of Carcinoma Cells in Effusions Using Monoclonal Antibodies

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

PI:	D. Solomon	Pathologist	LP NCI
OTHER:	A. Thor	Expert	LTIB NCI
	Y. Yee	Visiting Fellow	LP NCI
	E.W. Chu	Chief, Cytopathology Section	LP NCI
	J. Schlom	Chief, Laboratory of Tumor Immunology and Biology	LTIB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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.)*

The cytologic diagnosis of metastatic adenocarcinoma in effusions can be very difficult. Not only can malignant cells have very bland cytologic features but reactive mesothelial cells can assume a very atypical appearance. Using the avidin-biotin immunoperoxidase technique on cytospin preparations of pleural and peritoneal effusions, we investigated the reactivity of purified monoclonal antibody B72.3 with benign and malignant effusions. Initial effusion specimens studied were carefully selected to include only cytologically malignant effusions from patients with a history of adenocarcinoma and cytologically benign effusions from patients with no history of adenocarcinoma. Of the 38 malignant effusions studied, 20-90% of the cells demonstrated strongly positive staining with B72.3. There was no detectable staining of cells in reactive effusions. We conclude that immunostaining with B72.3 is useful in the cytologic diagnosis of metastatic adenocarcinoma in effusions.

We are now continuing our investigations using second generation monoclonal antibodies raised against TAG-72 antigen. We also plan to extend the utilization of these antibodies to fine needle aspiration specimens.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To determine the efficacy of immunocytochemistry in the cytologic diagnosis of metastatic carcinoma.

Methods Employed:

Millipore filter preparations and Diff-quik stained cytospin preparations of thoracentesis and paracentesis specimens were evaluated on the basis of morphological criteria used. In addition, aliquots of each specimen were studied for immunocytochemical staining using monoclonal antibody B72.3 in the avidin-biotin immunoperoxidase assay. Purified mouse myeloma protein MOPC-21 at equivalent protein concentration was used as a negative control.

Major Findings:

Fifty-two specimens have been studied, including 30 pleural effusions and 18 ascites fluids. Of the 38 cytologically malignant effusions studied for reactivity with the B72.3 antibody, all contained cells which stained strongly and specifically except for one case of metastatic gastric cancer. The percent of malignant cells staining varied between 20 and 90%. In contrast, none of the cells in the reactive effusions stained with the B72.3 antibody.

Significance to Biomedical Research and the Program of the Institute:

While the value of immunoperoxidase to histopathology has been amply demonstrated, the application of immunocytochemistry to cytological specimens is only beginning to be explored. We have developed an assay which takes advantage of the fact that cytologic specimens consist of suspensions of viable cells. Rather than subject the tumor cells to rigorous processing, we make cytospin preparations. This approach offers a number of advantages: (1) it is rapid; (2) it is generally applicable; (3) a small number of cells is sufficient. Using this technology, the purified monoclonal antibody B72.3 has been shown to be sensitive and specific in aiding the cytologic diagnosis of malignancy in cytological effusions.

The combination of this methodology with fine needle aspiration cytology promises to provide a rapid, reliable, and inexpensive approach to the daily diagnosis of cancer.

Proposed Course of Research:

We plan to continue our investigation of monoclonal antibodies useful in the cytologic diagnosis of malignancy and to extend our studies to include fine needle aspiration material.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00545-08 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 chererters 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 standerd unreduced type. Do not exceed the spece 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.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

We wish to study extracellular matrix protein (ECM) synthesis by normal and tumor cells in vitro, in cultures established by us from patients with known disease and in lines obtained from commercial sources.

Methods Employed:

These lines will be carefully evaluated for tumor cell growth, using all available techniques (chromosome analysis, density dependent growth, tumor production in nude mice, etc.). Matrix protein synthesis will be evaluated by ion exchange chromatography, slab gel electrophoresis, autoradiography of radiolabelled cells, immunoprecipitation and SDS-PAGE, and electrophoretic transfer with immunodetection.

Major Findings:

1. Eleven Ewing's sarcoma cell lines have been established or investigated and have shown a unique collagen synthetic profile. These cells variably produce types I, III, and IV collagen. This pattern has not been observed in any other tumors or tumor cell lines studied or reported. 2. Five neuroblastoma lines have been studied; three are classic adrenal-origin tumors and synthesize no identifiable stromal collagens (ie, I or III), and are thus distinct from Ewing's sarcoma, which they otherwise resemble. Two of the lines are peripheral in origin, show some morphologic features of Schwann cells, and like those cells, synthesize some stromal collagens as well as FN, LM, and type IV collagen 3. Preliminary data from osteosarcoma, chondrosarcoma, fibrosarcoma, and carcinoma cultures and, in some cases, tumors, indicated characteristic collagen type synthesis - types I, II, III, and IV, respectively. 4. Laminin and fibronectin have been detected in some tumors but not others, in patterns which reflect ectodermal or mesodermal origin of various childhood tumors.

Significance to Biomedical Research and the Program of the Institute:

Study of in vitro synthesis of matrix components by human tumors will substantiate the applicability of similar studies in animal model systems. These results will 1) have bearing both on our understanding of the origin of these tumors (with obvious therapeutic implications) as well as 2) provide valuable models for the study of the synthesis and assembly of the extracellular matrix by human tissues, as well as providing isolated, purified components of human origin, whose structure and function can then be studied in detail.

Proposed Course of Research:

An appropriate array of tumors has been established in culture (sarcomas, neuroblastomas, Ewing's sarcomas, and lymphomas). Their patterns of matrix protein synthesis have been determined. This data has been presented in abstract form and is submitted for publication. Further work will focus on the effect of differentiation on ECM synthesis. Also, the character and biological activity of these newly described ECM constituents will be studied.

Publications:

Scarpa, S., Modesti, A., and Triche, T.J.: Extracellular matrix synthesis by undifferentiated childhood neoplasia. Am. J. Pathol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00874-04 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Neurone-specific Enolase in Childhood Tumors

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

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP NCI
OTHER:	M. Tsokos	Visiting Scientist	LP NCI
	R.I. Linnoila	Medical Staff Fellow	LP NCI
	R. Chandra	Children's Hospital, Washington, D.C.	

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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

A

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

The diagnosis, and thus therapy, of solid tumors of childhood is often difficult due to lack of distinguishing characteristics. This is especially true of Ewing's sarcoma, neuroblastoma, primitive soft tissue sarcomas, and (occasionally) lymphoma. We have evaluated the presence of a specific neural enzyme, neurone-specific enolase (NSE), in paraffin-embedded sections of a diverse group of solid childhood tumors, including previously unrecognized variants of neural tumors, employing immunocytochemistry with antisera to NSE. We find uniform reactivity of all neural tumors with this antibody. No cross-reactivity with non-neural tumors, save a rare example of differentiated rhabdomyosarcoma, was found. We conclude that NSE is a reliable, readily detected marker in even primitive childhood tumors of neural origin. Also, we have defined the neural histogenesis of a newly described, "round cell" tumor of chest wall resembling Ewing's sarcoma, the so-called Askin tumor, which in reality is a form of peripheral neuroepithelioma. Finally, we have recently confirmed the unique character of so-called peripheral neuroepithelioma, which is NSE-positive but which displays hybrid neural and Schwannian morphologic characteristics.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To evaluate the use of a neural marker (NSE) in the diagnosis of primitive solid childhood tumor diagnosis.

Methods Employed:

Paraffin sections of various childhood tumors are incubated with monospecific, anti-neurone-specific enolase raised against purified rat brain enolase ($\gamma\gamma$ isomer), and detected by the Petrusz immunoenzyme bridge technique.

Major Findings:

1. Only tumors of neural crest origin are reactive with the antibody, save for an isolated instance of reactivity with only the differentiated myoblasts of a rhabdomyosarcoma. 2. Normal skeletal muscle, some smooth muscle, nerve, and liver are also reactive. This may reflect cross-reactivity with the α or β isomer subunits, found in liver and muscle, respectively. 3. All neural crest tumors evaluated so far (>40) have been reactive. 4. Previously undescribed or ill-understood neoplasms have been studied and their normal histogenesis confirmed (or refuted). Specifically, the so-called Askin tumor of chest wall has been shown to be a peripheral neuroepithelioma.

Significance to Biomedical Research and the Program of the Institute:

A reliable means of identifying all neural crest tumors in ordinary tissue sections is, to date, unavailable. This technique appears to provide such means. This is critical in determining treatment and assessing results of treatment.

Proposed Course of Research:

This project is now being pursued in conjunction with monoclonal antibody studies of these tumors (See Z01 CB 00899-02 LP).

Publications:

Triche, T.J., Askin, F.B, and Kissane, J.M.: Neuroblastoma, Ewing's sarcoma, and the differential diagnosis of small-, round-, blue-cell tumors. In Finegold, M. (Ed.): Pathology of Neoplasia in Children and Adolescents. Philadelphia, W.B. Saunders Company, 1986, pp. 145-195.

Linnoila, R.I., Tsokos, M., Triche, T.J., Marangos, P.J., and Chandra, R.S.: Evidence for neural origin and PAS positive variants of the malignant small cell tumor of thoracopulmonary region ("Askin tumor"). Am. J. Surg. Pathol. 10: 124-133, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09125-03 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Cytogenetic Abnormalities and Oncogene Expression of Small, Round Cell Tumors

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

PI:	M. Israel	Senior Investigator	PB NCI
OTHER:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP NCI
	C. Thiele	Research Associate	PB NCI
	J. Whang-Peng	Chief, Cytogenetic Oncology Section	MB NCI
	E. Gelmann	Senior Investigator	LTCB NCI
	J. Miser	Expert	PB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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 encountered a uniform rcp (11:22) translocation in Ewing's sarcoma. This is true of all lines and tumors examined to date (~20). It is not true of neuroblastoma, lymphoma, or soft tissue sarcoma. Interestingly, it is also present in a unique childhood tumor, peripheral neuroepithelioma and the closely related chest wall tumor described by Askin et al., the so-called Askin tumor. The break point on chromosome 22 is close to a known oncogene, c-sis. No amplification or rearrangement of c-sis has been detected. In the case of peripheral neuroepithelioma, c-sis is not amplified, but c-myc is. Unlike classic neuroblastoma, N-myc is not expressed. These results serve to emphasize the common abnormality found in Ewing's sarcoma, its distinction from other round cell tumors, and the unique character of peripheral neuroepithelioma.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

1. To detect reproducible chromosomal abnormalities in solid tumors of childhood. 2. To relate oncogene expression to specific tumor types, and potentially to tumor aggressiveness. 3. To detect the relationship, if any, between oncogene amplification, translocation, or rearrangement and *in vivo* or *in vitro* malignancy of a given tumor.

Methods Employed:

1. Cell lines and fresh tissue from patients with Ewing's sarcoma, neuroblastoma, soft tissue sarcoma, or related tumors. 2. Cytogenetics and *in situ* hybridization with various radiolabelled cDNA probes(λ, n-myc, c-myc, c-sis). 3. Southern blots of high molecular weight cell DNA.

Major Findings:

1. All Ewing's sarcoma lines and tumors examined to date possess a reciprocal (11:22) translocation. 2. This translocation of the long arm of 22 occurs close to c-sis, a known oncogene encoding mRNA for platelet derived growth factor. 3. No amplification or rearrangement of c-sis has been detected to date. 4. This abnormality is found in no other solid tumor of childhood, save for peripheral neuroepithelioma.

Significance to Biomedical Research and the Program of the Institute:

Recent results in many laboratories have established the association of oncogene expression with specific tumors. N-myc has been implicated in class II (fatal) neuroblastoma. No such relationship has been detected for other childhood tumors. We are attempting to determine similar relationships between known oncogenes and other tumors. The ability to detect these oncogenes may have important diagnostic, prognostic, and therapeutic implications.

Proposed Course of Research:

More tumors of diverse type will be studied for the presence of specific cytogenetic abnormalities. The expression of certain oncogenes, especially n-myc, c-myc, and c-sis and related oncogenes will be assessed by a variety of techniques.

Publications:

Whang-Peng, J., Triche, T.J., Knutsen, T., Miser, J., Kao-Shan, S., Tsai, S., and Israel, M.A.: Cytogenetic characterization of selected small round cell tumors of childhood. *Cancer Genet. Cytogenet.* 21: 185-208, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09132-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

The Role of Laminin and its Receptor in Differentiation of Neuroblastoma

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

PI:	M. Tsokos	Visiting Scientist	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP NCI
	M.E. Sobel	Senior Investigator	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	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have recently studied the synthesis and deposition of the extracellular matrix (ECM) proteins laminin (LM), fibronectin (FN) and type IV collagen by human neuroblastoma (NB) cells in vitro, before and after differentiation with dibutyryl-cyclic (dbc) AMP and retinoic acid (RA). The synthesis of all the above ECM proteins was confirmed by polyacrylamide gel electrophoresis (PAGE) of the conditioned culture media of several NB cell lines, after metabolic radiolabelling with 3H-leucine and 3H-proline. Deposition of the proteins on the cell layers was confirmed by immunofluorescence (IF), using antisera against LM, FN and type IV collagen on the cell layers. The pattern of expression of the above proteins by specific morphologic phenotypes suggested to us that these proteins may play a role in neuronal-Schwann cell interaction and hence differentiation of NB. Specifically, the fact that LM was only present on the surface and the cytoplasm of flat cells with Schwannian characteristics and absent from neuronal cells, led us to believe that: (1) cells with features of Schwann cells and neurones were appearing in culture and (2) these cells might recapitulate normal Schwann cell-neurone interaction. In this case, the abundant cell surface LM on Schwann cells might be a chemoattractant for LM-poor, LM-receptor-rich neuronal cells. The existence of such a receptor to laminin has been recently confirmed by several investigators for breast carcinoma, fibrosarcoma and muscle cells (Rao et al., Terranova et al., Lesot et al., and Malinoff and Wicha). Moreover, other authors have shown that neurons extend neurites on laminin substrata. To evaluate the validity of this hypothesis, (1) we have searched for the presence of a laminin receptor in NB cell lines, its exact localization in specific cell phenotypes, before and after neuronal and/or Schwannian differentiation using a monoclonal antibody against the LM receptor and (2) we have searched for possible differences in the expression of the mRNA which is involved in the synthesis of the LM receptor.

Project Description

Objectives:

To determine the biologic mechanisms of differentiation of NB into neurones and Schwann cells, which may eventually explain similar mechanisms of normal embryonic development of the nervous system as well.

Methods Employed:

Tissue culture, electron microscopy, immunofluorescence, immunoperoxidase, total cellular RNA isolation, Northern blot, hybridization.

Major Findings:

(1) NB cells express small amounts of LM receptor which increase considerably after neuronal and, to a lesser degree, Schwannian differentiation. Moreover, the LM receptor is predominantly localized in the neuritic processes when the NB cells differentiate into a neuronal phenotype.

(2) The message for the LM receptor increases considerably after neuronal differentiation.

Significance to Biomedical Research and the Program of the Institute:

Clarification of mechanisms of differentiation of NB cells *in vitro* is important to understand the biologic behavior of this tumor *in vivo* and possibly establish new methods of treatment.

Proposed Course of Research:

These preliminary results support the existence of a laminin receptor in primitive NB cells. A more vigorous proof of their existence by additional experiments and an exact quantitative evaluation before and after differentiation are necessary subsequent experiments to elucidate the role of LM and LM receptors, if any, in neural development and differentiation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09133-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Neuroblastoma: The Effect of Morphologic Transformation on Metastatic Potential

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

PI:	M. Tsokos	Visiting Scientist	LP NCI
OTHER:	S. Scarpa	Visiting Fellow	LP NCI
	J. Williams	Biologist	LP NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI
	S.J. Mims	Biologist	LP NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP NCI

COOPERATING UNITS *(if any)*

R. Ross, Department of Biological Sciences, Fordham University, Bronx, New York

LAB/BRANCH

Laboratory of Pathology

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Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We treated a classic neuroblastoma (NB) and a peripheral neuroepithelioma (PN) with the differentiating agents dibutyryl-cyclic (dbc) AMP and retinoic acid (RA) and evaluated the influence of several factors, such as morphologic transformation, biochemical expression, cell growth, total protein synthesis and extracellular matrix (ECM) protein synthesis to their metastatic capacity in vivo. The latter was evaluated by injecting treated and untreated cells from both lines in the tail vein of 31 nude mice. The mice were sacrificed after the injection and complete autopsies were performed.

The results showed that the same differentiating agents favored a diverse pathway of differentiation in the 2 studied lines. Moreover, Schwannian transformation was associated with a remarkable increase in total protein synthesis and ECM protein synthesis and no changes in cell growth, whereas neuronal transformation was accompanied by a minor increase in total protein synthesis, decrease in ECM protein synthesis and inhibition of cell growth. Schwannian transformation was associated with a higher metastatic potential, when compared to untreated or neuronally transformed cells. The explanation of the latter phenomenon is not known. However, high levels of LM and unsaturated receptors have been alternatively implicated in the high metastatic potential of other cell types and we intend to look for LM and its receptors in the nude mice tumors from metastatic sites.

Project Description

Objectives:

To determine factors associated with a high metastatic potential of NB cells.

Methods Employed:

Tissue culture, cell differentiation *in vitro*, polyacrylamide gel electrophoresis, immunofluorescence, electron microscopy, and tail vein injection of tumors in nude mice.

Major Findings:

(1) Differentiation of classic NB and P.N. into specific cell types *in vitro* (neuronal, Schwannian) is associated with specific biochemical changes, i.e. increase of C.A.T. in neuronal and C.N.P. in Schwannian transformation and increased protein synthesis including increased synthesis and deposition of LM, FN and type IV collagen in Schwannian and decreased in neuronal transformation.

(2) Schwannian transformation is associated with a higher metastatic potential *in vivo*.

Significance to Biomedical Research and the Program of the Institute:

Neuroblastoma is a tumor with variable clinical behavior; factors such as age, stage, and histologic maturation play important roles. Most neuroblastomas are refractory to current treatment. The understanding of mechanisms of metastasis of this tumor is a major goal of pediatric oncology, since it may help identify new treatment modalities. Moreover, histologic evaluation of tumors with more sophisticated techniques, such as detection of LM receptors or LM itself on sections may be of great prognostic value and possibly an indicator of specific treatment in the future.

Proposed Course of Research:

The original results will be confirmed by repeating the experiment with a new batch of nude mice. In addition, the tumors from the mice from both primary and metastatic sites will be frozen and frozen sections will be stained by immunofluorescence using a LM-FITC ligand and anti-LM antibody to evaluate the presence of LM and LM receptors on the cells of primary and metastatic sites. Samples of the tumor will also be evaluated by electron microscopy for detection of subtle morphologic changes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09135-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Peripheral Neuroepithelioma - A Tumor Distinct from Neuroblastoma

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

PI:	M. Tsokos	Visiting Scientist	LP NCI
OTHER:	M.A. Israel	Senior Investigator	PB NCI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP NCI

COOPERATING UNITS *(if any)*

L. Donner, Resident, George Washington University, Washington, D.C.
C.P. Reynolds, Transpl. Res. Program, National Naval Medical Center, Bethesda, MD

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

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

A

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

Peripheral neuroepithelioma (PN) has been underdiagnosed for many years, because of its histologic resemblance to Ewing's sarcoma in most cases, or occasionally to rhabdomyosarcomas (RMS). Lately, we were able to prove the neuroectodermal nature of the small round cell tumor of thoracopulmonary region ("Askin tumor"), a variant of PN, employing immunoperoxidase to detect the presence of the enzyme neuron-specific enolase (NSE) and electron microscopy (EM). In a wider study of small round cell tumors of childhood, we also found that NSE was only present in neuroectodermal tumors and not in Ewing's sarcoma or lymphoma. However, rhabdomyosarcomas (RMS) were also nonspecifically positive for NSE, and therefore, for distinction of RMS from PN, muscle markers and EM are additional diagnostic tools.

Here we evaluated the histologic, immunocytochemical, cytochemical and EM features of 15 cases of PN and correlated them with clinical presentation. All tumors were extra-adrenal and occurred in diverse soft tissue and bony sites, in post-ganglionic locations. All patients were older than these in classic neuroblastoma (NB) and secreted no catecholamines. All tumors were NSE positive and 50% S-100 protein positive. The EM appearance was overall more primitive but the neural characters were established in most cases. Immunofluorescence with monoclonal antibodies showed a specific pattern of staining for PN, distinct from NB and similar, although not identical to Ewing's sarcoma.

GPO 914-918

Project Description

Objectives:

To establish firm morphologic and immunocytochemical criteria for distinction of the entity peripheral neuroepithelial from classic neuroblastoma, Ewing's sarcoma and rhabdomyosarcoma.

Methods Employed:

Light microscopy, electron microscopy, immunoperoxidase and immunofluorescence.

Major Findings:

(1) Peripheral neuroepithelioma, although it may resemble Ewing's sarcoma or rhabdomyosarcoma by light microscopy, has a unique EM appearance in most cases and expresses NSE immunoreactivity, whereas Ewing's sarcoma is negative for NSE and rhabdomyosarcoma and exhibits muscle markers.

(2) Peripheral neuroepithelioma is different from classic neuroblastoma, in that it presents in an older group of patients, in extra-adrenal, postganglionic sites, does not secrete catecholamines, exhibits less morphologic differentiation to neuroblasts, shows more often other neuroectodermal cell populations, such as Schwann cells and melanocytes and exhibits a specific pattern of monoclonal antibodies positive for HLA-class I base determinants (W6/32) and class I associated antigen (BBM.1) and equivocally positive for the neuroblastic antigen HSAN 1.2, in contrast to classic neuroblastoma.

(3) Peripheral neuroepithelioma is intermediate in its reactivity of monoclonal antibodies between classic neuroblastoma and Ewing's sarcoma.

Significance to Biomedical Research and the Program of the Institute:

The clinical course and therapeutic responsiveness of peripheral neuroepithelioma is unknown, since this tumor has been masked so far with other diagnoses, due to absence of firm histologic criteria. The establishment of diagnostic tools for distinction of this entity from other small round cell tumors of childhood has been a necessity for the clinical pediatric oncology group at the NCI, since it will lead to a better understanding of its clinical behavior and prognosis, as well as to institution of the best possible treatment for these tumors. Moreover, prospective distinction of this tumor from Ewing's sarcoma will answer the question if they are indeed different clinical entities, or one is the primitive form of the other.

Proposed Course of Research:

The project is almost completed. Minor additional studies will include detection of choline acetyltransferase and neurofilaments by immunofluorescence, using specific monoclonal antibodies on the 4 peripheral neuroepithelioma cases that we have frozen tissue on, as well as immunostaining of some more Ewing's sarcoma cases with NSE to confirm the specificity of the latter enzyme in the distinction of these two tumors.

Publications:

Linnoila, R.T., Tsokos, M., Triche, T.J., Marangos, P.J., and Chandra, R.S.: Evidence for neural origin and PAS positive variants of the malignant small cell tumor of thoracopulmonary region ("Askin tumor"). Am. J. Surg. Pathol. 10: 124-133, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09137-02 LP

PERIOD COVERED: October 1, 1985 to September 30, 1986

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.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To determine the histogenesis of Ewing's sarcoma and its relationship to other childhood tumors.

Methods Employed:

Tissue culture, electron microscopy, immunocytochemistry with monoclonal and polyclonal antibodies, catecholamine fluorescence, biosynthetic radiolabel incorporation studies of protein synthesis.

Major Findings:

1. Ewing's sarcoma can be induced to develop neural phenotypic characteristics following treatment with appropriate differentiating agents in vitro.

2. This neural phenotype closely resembles that expressed by so-called peripheral neuroepithelioma, but not classic childhood neuroblastoma.

Significance to Biomedical Research and the Program of the Institute:

The proper classification and treatment of round cell, primitive tumors, of which Ewing's sarcoma is the most conspicuous example, is entirely empirical to date. Rational treatment of this and related tumors depends on a basic understanding of their inter-relationships and origins. Such information has not been available for these tumors in the past, but is becoming available as a result of these studies.

Proposed Course of Research:

These initial, but very promising, results must be confirmed by more vigorous proofs. Additional studies employing diverse other techniques beyond morphology and immunocytochemistry are underway.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09138-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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 frgments 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.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To determine whether N-myc expression on a cell-by-cell basis can be correlated with clinical outcome more precisely than existing studies on average N-myc expression by whole extracts of tumor DNA.

Methods Employed:

In situ hybridization of radiolabelled N-myc probe and immunodetection of N-myc protein in frozen sections of approximately 80 cases of childhood neuroblastoma obtained from one of us (RS). Autoradiographic detection and morphometric analysis of grains, and correlation with tumor cell morphology, will be employed.

Major Findings:

This study is in its early stages. To date, the approach has been proven technically feasible, and N-myc positive and negative tumors have been successfully distinguished. Study of the patient tumor population noted above is underway.

Significance to Biomedical Research and the Program of the Institute:

Neuroblastoma is a tumor refractory to treatment in most cases, for unknown reasons. The ability to identify such cases prior to treatment, and to understand why they are not amenable to treatment, is a major goal of pediatric oncology, both at the NCI and at every major pediatric oncology program.

Proposed Course of Research:

The 80 tumors noted above, as well as new cases received during the course of the study, will be studied as outlined above. Correlation with morphology of the frozen sectioned tumor, as well as other material available on the case, will be undertaken. Prognostically important variables will be ascertained. Additional studies with other tumors and other oncogenes are anticipated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09139-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Monoclonal Antibodies to Ewing's Sarcoma

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

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section	LP NCI
	S. Arakawa	Fogarty Fellow	LP NCI
	C.P. Reynolds	Tissue Transplantation Unit	NMC NCR

COOPERATING UNITS *(if any)*

Tissue Transplantation Unit, NMC, NCR

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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 standerd unreduced type. Do not exceed the space provided.)*

This is a new project, just commencing. Extensive studies of Ewing's sarcoma and related round cell tumors of childhood have served to distinguish most from one another on the basis of positive reactivity with one or more antibodies. No antibody specific for Ewing's sarcoma, or restricted to a few tumors including Ewing's, has been identified to date. It is the aim of the present study to produce a battery of monoclonal antibodies against selected, well-characterized Ewing's sarcoma lines established in this laboratory. They will then be studied and compared in their reactivity with other monoclonal antibodies previously reported. Any antibody found to be strongly reactive with Ewing's sarcoma cells will be further characterized, with the eventual intention to use same as a potential diagnostic, imaging, and therapeutic tool.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To create an antibody or antibodies which will identify Ewing's sarcoma cells.

Methods Employed:

Tumor cell lines, hybridoma technology, fluorescence activated cell sorter, immunofluorescence.

Major Findings:

Two monoclonal antibodies from a fusion of cells primed with TC-71 tumor cells have shown broad reactivity with numerous neoplastic, but not normal cells.

Significance to Biomedical Research and the Program of the Institute:

The lack of a means to positively identify Ewing's sarcoma cells, *in vivo* or *in vitro*, has hampered efforts to precisely define this enigmatic entity. Further, therapeutic strategies such as bone marrow purging have been impossible, due to the lack of a means of identifying tumor cells other than by morphology. Even morphology is nonspecific; no specific feature of Ewing's sarcoma has even been identified. Thus, the availability of a positive identifier of this tumor is much needed for diagnosis and treatment purposes.

Proposed Course of Research:

Spectrum of reactivity by tumor type will be catalogued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09140-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

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Ultrastructural Pathology Section

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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 unraduced 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

Project Description

Objectives:

To determine the identity and biologic function of a newly described protein suspected of being an extracellular matrix protein.

Methods Employed:

Tissue culture, biosynthetic radiolabel incorporation, gel permeation and high performance liquid chromatographic purification of conditioned medium proteins, electrophoretic purification, selective degradation studies, monoclonal antibody generation, and tissue immunocytochemistry of normal and neoplastic tissues, using such monoclonal antibodies.

Major Findings:

We have identified a previously undescribed, very high molecular weight (500,000 D), single-stranded (>700 nm) protein which is distinct from all known extracellular matrix proteins, but which is intimately associated with laminin, type IV collagen, and fibronectin in conditioned tissue cultured medium. The function and relationship to known extracellular matrix proteins is under investigation.

Significance to Biomedical Research and the Program of the Institute:

Studies of tumor cell biology, especially the degradation of pericellular extracellular matrix, have focused on the role of discrete elements of the extracellular matrix, such as laminin, in moderating the biologic behavior (manifest as invasion and/or metastasis) of malignant cells. These studies require a detailed and sophisticated knowledge of the structural organization and composition of the extracellular matrix. Such knowledge has only recently begun to accumulate, and is still incomplete. Studies such as this will provide a far more comprehensive picture of how the individual elements of the pericellular matrix are organized, and their function in normal and neoplastic tissues.

Proposed Course of Research:

This project is nearly completed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09143-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Differentiation of Human Retinoblastoma

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

PI:	M. Tsokos	Visiting Scientist	LP NCI
OTHER:	A.P. Kyritsis	Visiting Associate	LVR EI
	G.J. Chader	Chief, Laboratory of Vision Research	LVR EI
	T.J. Triche	Chief, Ultrastructural Pathology Section	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20902

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.)*

Monolayer cultures of the Y-79 human retinoblastoma cell line were obtained recently by growing the cells on substrata specifically covered with poly-O-lysine and fibronectin. The cells thus attached were studied by light and electron microscopy, immunofluorescence and immunoperoxidase before and after differentiation with various agents, such as dibutyryl-cyclic AMP (dbc-AMP) and sodium butyrate (Nabut). Serum-supplemented and serum-free media were used. Mere attachment of the cells resulted in minor degrees of morphologic differentiation, which was enhanced after the addition of the agents. Round cells with processes, as well as flat, substrate-adherent cells appeared. Specific neuronal and glial markers were expressed by the cells in various conditions. Moreover, ultrastructural characteristics of neuronal, photoreceptor, glial and pigmented cells were observed. The results support differentiation of primitive retinoblastoma cells, such as those of the Y-79 line, into various cell types of normal human retina, i.e. photoreceptor, neuronal, Müller and pigmented epithelial cells. These findings not only support origin of retinoblastoma from a primitive cell of the optic disc, but also offer a system to study cell interactions and tumor cell responses to various therapeutic agents.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To investigate the histogenesis of human retinoblastoma, and to establish a system of cell interaction, which will help to understand developmental mechanisms of human retina.

Methods Employed:

Tissue culture differentiation experiments, immunofluorescence, immunoperoxidase, electron microscopy.

Major Findings:

1. Retinoblastoma cells can attach on substrates covered with poly-D-lysine and fibronectin.

2. Retinoblastoma cells differentiate into photoreceptors, neuronal, glial and pigmented epithelial cells. The pathway of differentiation can be controlled by specific agents.

3. Neuronal and/or glial cell markers are expressed by specific cell types, after differentiation. The neuronal or photoreceptor cell markers detected in the cells include choline-acetyl transferase (C.A.T.), neuron-specific enolase and interphotoreceptor protein whereas the glial markers include glial fibrillary acidic protein and myelin basic protein.

4. By electron microscopy, several specific morphologic characteristics were seen: a) rosette formation with microvillous processes towards the lumen and occasional centrioles (photoreceptor cell differentiation); b) long cell processes with neurosecretory granules (neuronal differentiation); c) cells with darker cytoplasm, intermediate filaments and well-developed intercellular attachments (glial cell differentiation); d) cells with melanosomes (pigmented epithelial cells).

Significance to Biomedical Research and the Program of the Institute:

The differentiation of retinoblastoma appears to be analogous to neuroblastoma. Because both tumors are neuroectodermal and have many other features in common, the findings in these tumors complement each other and help establish a unifying concept of differentiation and biologic behavior of neuroectodermal tumors in general. New methods of treatment may be the result of such studies.

Proposed Course of Research:

Mechanisms of interactions among the emerging cell types will be studied. As an immediate step, we will study the expression of laminin receptor by the retinoblastoma cells and find if we can control the expression of this receptor manipulating the conditions of the culture to induce specific types of differentiation.

Publications:

Tsokos, M. and Kyritsis, A.P.: Neuroblastoma, retinoblastoma, medulloblastoma: Primitive neuroectodermal tumors with common characteristics. A review. Mat. Med. Greca 12: 519-531, 1984.

Kyritsis, A.P., Tsokos, M., and Chader, G.J.: Behavior of retinoblastoma cells in tissue culture. In Chader, G.J. and Osborne, M.N. (Eds.): Progress in Retinal Research. London, Academic Press, 1986 (in press)

Tsokos, M., Kyritsis, A.P., Chader, G.J., and Triche, T.J.: Differentiation of human retinoblastoma in vitro into cell types with characteristics observed in embryonal or mature retina. Am. J. Pathol. (in press)

Kyritsis, A.P., Tsokos, M., Triche, T.J., and Chader, G.J.: Retinoblastoma: A primitive tumor with multipotential characteristics. Invest. Ophthalmol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00523-07 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Complex Carbohydrate Released from Mammalian Cells by Trifluoroacetolysis

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

PI:	D.A. Zopf	Chief, Biochemical Pathology Section	LP NCI
OTHER:	G.C. Hansson	Visiting Fellow	LP NCI
	J. Cashel	Biologist	LP NCI

COOPERATING UNITS *(if any)*

R.S. Shirey, Technical Specialist, Blood Bank, Johns Hopkins Hospital, Baltimore, MD

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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.)*

Carbohydrate chains released by trifluoroacetolysis of whole tissues, tissue fractions, or cells grown in culture, are easily recovered in nearly quantitative yield and reconstituted to their native form. Analysis of the majority of oligosaccharides containing six or fewer monosaccharide units is performed by combined gas chromatography and mass spectrometry of permethylated, N-trifluoroacetylated oligosaccharide derivatives. Analysis for certain specific oligosaccharides is carried out by radioimmunoassay using antibodies produced against purified oligosaccharides coupled to polypeptide carriers. It is anticipated that the repertoire of oligosaccharide chains produced by cells or tissues will reflect states of cellular differentiation and reveal potential cell surface markers.

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

Project Description

Objectives:

To separate and identify the major oligosaccharide chains present in mammalian cells and to correlate the occurrence of specific oligosaccharide structures with states of cellular differentiation.

Methods Employed:

Maternal-fetal incompatibility within the P blood group system has been implicated as a cause of repeated spontaneous abortion. The P blood group antigen has been identified by Marcus and his collaborators as the glycosphingolipid globoside (GalNAcβ1-3Galα1-3Galβ1-4Glc-Cer), the major glycolipid of human red cells. Rare individuals with the p_1^k phenotype have red cells that lack globoside and instead contain increased amounts of trihexaosylceramide (Galα1-3Galβ1-4Glc-Cer), the immediate biosynthetic precursor to globoside.

We have studied a female patient of blood group p_1^k whose husband belongs to blood group P. The patient had suffered 13 consecutive spontaneous abortions, all before 12 weeks gestation. The patient's fourteenth pregnancy was maintained by plasmapheresis (3 X per week) to reduce her circulating anti-P titer. After successful delivery of a live infant at 33 weeks, we obtained the placenta and extracted glycolipids. Their structure were determined by trifluoroacetolysis and combined GC/MS. Reactivities of maternal serum antibodies with placental glycolipids was determined by radioimmuneoverlay of glycolipids separated on thin-layer plates.

Major Findings:

The major neutral glycolipids from the placenta of the blood group P child delivered from the p_1^k patient under study were globoside, trihexaosylceramide, and lactosylceramide. The same glycolipids were identified in a normal control placenta from an infant of blood group P. Antigloboside antibodies of IgM, IgA, and IgG subclasses were identified in the maternal serum obtained by plasmapheresis. The very simple structures found in glycolipids of the placenta contrast with much more complex glycolipids known to occur in many other fetal tissues including erythrocytes and intestine.

Significance to Biomedical Research and the Program of the Institute:

The simple glycolipids of placental trophoblast contain none of the blood group-active or other oncofetal carbohydrate antigens such as A, B, SLe^a, etc. whose expression can vary widely in the population. Thus, the maternal immune system seldom encounters trophoblast-associated fetal glycolipid immunogens. A single reported case of tumor regression of a gastric carcinoma following an immune crisis induced by blood group P transfusion mismatch has been reported. The present findings account for the chronic spontaneous abortion syndrome in p_1^k individuals on a chemical basis and suggest that antiglycolipid immune responses might be useful as mediators of tumor immunotherapy.

Proposed Course of Research:

Studies of glycolipid immunogens from normal and malignant tissues will continue. Glycolipid compositions of cells that are "tolerated" in multiple tissue sites (e.g., lymphocytes) will be compared to glycolipids of placenta and to glycolipids of other cell types whose localization is more highly tissue specific (e.g., gastrointestinal epithelium).

Publications:

Hansson, G.C. and Zopf, D.A.: Biosynthesis of the cancer-associated sialyl-Le^a antigen. J. Biol. Chem. 260: 9388-9392, 1985.

Zopf, D. and Hansson, G.: The chemical basis for expression of the sialyl-Le^a antigen. In Wu, A. (Ed.): Molecular Immunology of Complex Carbohydrates. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00525-07 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Analysis of Oligosaccharides by Combined Gas Chromatography-Mass Spectrometry

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

PI:	D.A. Zopf	Chief, Biochemical Pathology Section	LP NCI
OTHER:	J. Cashel	Biologist	LP NCI
	E.A. Kabat	Consultant	IRP NIADDK
	J. Dakour	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

A. Lundblad, Head Physician, University of Lund, Sweden

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology 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

B

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

Separation of reduced and permethylated oligosaccharides by gas chromatography can be facilitated by the use of a fused silica capillary column coated with methyl silicon. The presence of N-acetylhexosamines in oligosaccharides increases their retention time and interferes with efficient GC separation. Transamidation of hexosamines by trifluoroacetolysis followed by reduction, removal of O-trifluoroacetyl groups and permethylation, dramatically reduces the retention time of hexosamine-containing oligosaccharides and permits separation of oligosaccharides containing up to six monosaccharide units, regardless of how many of these are hexosamines. The mass spectra of permethylated oligosaccharides with N-trifluoroacetylated amino sugars show unexpectedly high abundances of mass ions containing the N-trifluoroacetyl group. As many of these ions are large, they provide useful information regarding oligosaccharide structure.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

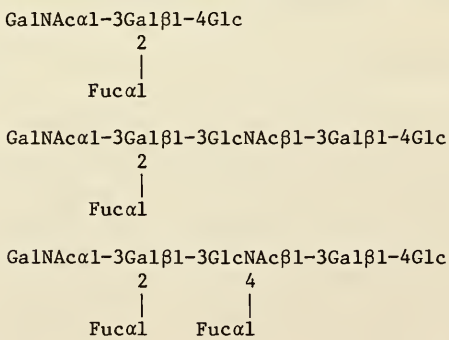
To develop methods for isolation of oligosaccharides and for their separation and analysis by gas chromatography and mass spectrometry.

Methods Employed:

Blood group-active oligosaccharides from human mucins are isolated and fractionated by affinity chromatography on columns containing monoclonal antibodies bound to sepharose 4B. Following trifluoroacetolysis, oligosaccharides are treated with sodium borohydride and methanolic ammonia and finally are permethylated. N-trifluoroacetylated and permethylated oligosaccharide alditols are separated by gas chromatography using a fused silica capillary column coated with methyl silicon. The column effluent is passed without separation into a mass spectrometer.

Major Findings:

Oligosaccharides can be separated by chromatography on immunoaffinity columns containing monoclonal antibodies bound to sepharose. Standard blood group A-active oligosaccharides purified from human urine with the structures:



can be separated from non-A-active oligosaccharides and from one another according to their different affinities for anti-A monoclonal antibodies. Following alkaline borohydride degradation of A-active human mucins, at least four A-active oligosaccharide components are isolated by the same method. Together, these comprise approximately 5% of the released oligosaccharides. Thus, immunoaffinity chromatography appears to offer a powerful new approach to purification of antigenically active carbohydrate chains from a complex mixture containing many structurally related but antigenically distinct oligosaccharides. Similar results have been obtained using anti-Le^a and anti-Le^b monoclonal antibodies and oligosaccharides with the respective antigenic activities.

Significance to Biomedical Research and the Program of the Institute:

A major technical problem in studying the structures of mucins from normal and malignant tissues has been the difficulty of separating and analyzing a minority (~5%) of antigenically active sugar chains from a large amount of structurally related but antigenically inactive material. Affinity chromatography using monoclonal antibody enables one-step isolation and partial characterization of antigenically active chains. This method should help enormously in structural elucidation of oncofetal antigens on tumor mucins and in studies of biosynthesis of tumor antigens.

Proposed Course of Research:

Oligosaccharides will be isolated after alkaline borohydride degradation of mucins obtained from normal fetal and adult tissues and from human tumors, especially colorectal and pancreatic carcinomas. Oligosaccharides that express A,B,H,Le^a,Le^b, and SLe^a antigens will be isolated by affinity chromatography and their structures will be determined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00556-04 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Expression of Glycolipids in Lymphocyte Subpopulations

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

PI:	D.A. Zopf	Chief, Biochemical Pathology Section	LP NCI
OTHER:	K. Schroer	Senior Assistant Surgeon	LP NCI
	M. Duk	Visiting Fellow	LP NCI
	J. Phung	Biologist	LP NCI
	J. Cashel	Biologist	LP NCI
	J. Fernandez	Biological Laboratory Technician	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	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.)*

Neutral glycolipids are differentially expressed in functionally distinct subpopulations of murine lymphocytes. Subpopulations of B cells can be studied by examining hybridoma lines derived from fusion of splenic B lymphocytes with the mouse myeloma SP2/0. We are analyzing total neutral glycolipids from hybridomas by thin layer chromatography and by GC/MS analysis of oligosaccharides after trifluoroacetolysis. Hybridomas from Balb/c splenocytes express glycolipids containing from two to five simple sugars. These include globoside and its precursors as well as asialo-GM2 and 2' fucosyllactosyl ceramide. The goal of this project is to correlate expression of oligosaccharide chains of glycolipids with functional parameters of B cell subsets such as responsiveness to Type I and Type II antigens.

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

Project Description

Objectives:

To examine the major glycolipids of hybridomas derived under conditions selective for early and late maturing B cell subsets.

Methods Employed:

A mouse myeloma (SP2/0) that expresses only mono- and dihexosylceramides is fused to mouse splenocytes. The fusion products express neutral glycolipids of both the myeloma and lymphocyte parent cells. Thus, glycolipids larger than dihexosyl ceramide are contributed by the B cell parent lymphocyte. Hybridomas (10^7 cells) are labeled in vitro for 48 h with [^{14}C]Gal and [^{14}C]GlcNAc and purified total neutral glycolipids are separated by thin layer chromatography and detected by autoradiography. Selected clones are expanded to 10^9 cells and the purified total neutral glycolipids are subjected to trifluoroacetolysis to release the oligosaccharide chains. The oligosaccharide mixture is reduced, permethylated, and analyzed by GC/MS.

Major Findings:

The composition of neutral glycolipids produced by cloned hybridomas derived from normal, healthy, immunologically "unstimulated" mice varies widely: a few hybridoma clones produce only lactosylceramides, whereas others produce various mixtures of higher glycolipids including globotriaosyl- and globotetraosylceramides, gangliotriaosyl- and gangliotetraosylceramides, 2'fucosyllactosylceramide (2'FLcer), and some higher fucosylated glycolipids whose oligosaccharide structures are not yet fully characterized. In contrast, a panel of IgM-secreting hybridomas prepared from splenocytes of LPS-treated Balb/cJ mice all produce identical glycolipids that include lactosyl ceramide, 2'FLcer, and globotriaosylceramide. A panel of anti-dextran secreting hybridomas from mice immunized with the polysaccharide antigen Dextran 1355S all produce identical neutral glycolipids that include the same structures seen in hybridomas from LPS-treated animals plus globoside. Anti-TNP secreting hybridomas from splenocytes of Balb/cJ mice immunized with TNP-KLH display a characteristic uniform pattern of glycolipids that includes a large amount of 2'FLcer.

Since 2'FLcer had not been described in lymphoid cells prior to these observations, we explored its biosynthesis and possible occurrence in mouse B and T cells, plasma cells, lymphomas, and plasmacytomas. Very small amounts of 2'FL, not detected in our initial studies, were observed in the myeloma cell line SP2/0 and in the precursor cell lines from which it was derived, P3X8 and MOPC 21. Other Balb/c myelomas also contain 2'FLcer, some in large amounts. In contrast, only trace amounts of 2'FLcer appear in B lymphocytes purified by panning Balb/c splenocytes on anti-immunoglobulin coated petri plates. Analysis of 2'FLcer in Balb/c plasma cells is in progress.

Studies on the biosynthesis of 2'FLcer indicate that the fucosyltransferase that catalyses the reaction:

$$\text{Gal}\beta1\text{-4Glc-cer} \xrightarrow[\text{GDP-Fuc}]{} \text{Fuc}\alpha1\text{-2Gal}\beta1\text{-4Glc-cer}$$

is identical to the enzyme that catalyses biosynthesis of higher H-blood group active glycolipids containing terminal Fucα1-2Galβ1-4GlcNAc... sequences. Addition of fucose to Galβ1-4Glc-cer in vitro proceeds much more slowly than addition to lacto- lactoneo-, and gangliotetraosylceramides. Thus, the presence of large amounts of 2'FLcer in some hybridomas and plasmacytomas suggests that extremely high levels of Fucα1-2 to Gal fucosyltransferase exist in some subsets of late B cells and plasma cells. Some glycoproteins of these cells may likewise be fucosylated by this enzyme.

Significance to Biomedical Research and the Program of the Institute:

The complex interactions between cells of the immune system are mediated by numerous recognition events at cell surfaces. We are studying the major glycolipids expressed by lymphocyte subpopulations in order to determine whether any correlations can be made between the carbohydrate chains displayed on the cell surface and the functional state of the lymphocyte. Results thus far strongly suggest that subpopulations responsive to different classes of antigens express different glycolipids. If additional studies confirm these correlations, then specific glycolipids can be used as markers for stages of cellular differentiation and their possible roles in immunoregulatory pathways should be explored. Immunoregulation of anticarbohydrate responses will be critically important for understanding immune surveillance against tumors since many recent attempts to define oncofetal antigens by hybridoma antibodies have yielded antibodies that react with cell surface carbohydrates.

Proposed Course of Research:

Comparison of hybridomas from newborn, adult, and xid mice will be pursued. Hybridomas made against Type I and Type II antigens early and late in development and in defective and non-defective reciprocal crosses will be studied. Splenic lymphocytes will be selectively purified using hybridoma antibodies against specific glycolipids and their responsiveness to different classes of antigens will be determined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00879-03 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Nucleotide Sequencing of Hybridoma Antibodies of Vh-GAC Family

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

PI:	K.R. Schroer	Senior Surgeon	LP NCI
OTHER:	J. Phung	Biologist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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.)*

The mouse immune response to streptococcal group A carbohydrate (GAC) utilizes many different gene segments from a diverse array of precursor Vh genes. The gene family number is estimated at 10 and the diversity of response in normal mice at over 200 different antibodies. The response is clonally restricted in each mouse immunized, but each mouse appears to express a different clonally homogeneous antibody in its serum in spite of genetically common background. The common denominator of similarity in these antibodies is their usage of highly homologous Vh gene segments, and identical Dh, and Jh segments. The serum response to GAC is entirely deficient in CBA/N mice which makes this an ideal system in which to investigate possible constraints on gene segment utilization.

Recent evidence indicates that up to 5% of CBA/N B cells utilize the Vh-GAC gene segment(s) in their Ig rearrangements: thus, 5-10% of plasma cells are stainable by an anti-Vh^{gac} antisera, and 5% of serum antibodies are positive for this serologic Vh marker by radioimmunoassay. This high frequency of expression of Vh gene(s) is greater than has previously been reported with any other Vh specific antisera, and provides an ideal experimental system to probe the Vh repertoire of CBA/N mice by examination of their B-cell derived hybridomas for structural constraints on combinatorial use. It is likely that a Vh-gene with such a high rate of representation in mouse B cells would probably serve redundantly as the V region for many antibodies of different binding specificity when combined with different D, and J segments along with different L chains. Thus, the family of inulin (B-2→1 fructosan) binding proteins share the Vh-GAC V-gene but use different joining segments and L chains. This V-gene has only been observed in anti-carbohydrate antibodies of normal mice and its paradoxical high expression by CBA/N B cells which fail to respond to carbohydrate antigens remains unexplained.

GPO 914-918

Project Description

Objectives:

To compare the structural correlates of hybridoma antibodies with their antigenic specificities and serologically defined families.

Methods Employed:

Hybridoma derived cell lines making antibodies bearing Vh determinants of the GAC-J606 family derived from Balb/c and CBA/N mice will be used to obtain deduced sequences of heavy and light chains of their antibodies. The sequencing method is that of Sanger et al. using oligonucleotide primed cDNA synthesis on an mRNA template to generate nucleotide sequence of V_H, D_H, J_H and of V_K, J_K from which the amino acid sequence is obtained.

Major Findings:

1. Two non-antigen binding hybridoma antibodies identified as Vh-GAC+ by serology have been partially sequenced by primed synthesis dideoxynucleotide (Sanger) methods from a poly A+ mRNA template. These sequences show extensive (>85%) sequence homology with the published V-HGAC9 gene. Translation sequences show only two definite amino acid substitutions (both neutral). One of these is a dinucleotide transversion and the other is a second base transition mutation (G-A). Three other transitions are third base silent changes. The sequence data provisionally support the interpretation that the important epitopes recognized by the anti Vh-GAC antibody reside in the Vh gene(s) product since these antibodies were selected without antigen and confirm the findings of previous workers with actual structures. That 5-10% of mouse B cells utilize this family of V genes makes this a very interesting and accessible gene family by this immunoselection procedure. No actual assignment of allelism can be made from these sequences even though they are highly homologous.

2. A comparison of the sequences of the three joining areas of the Vh-GAC+ antibodies shows no resemblance of their Dh or Jh segments or their junctionally encoded amino acids to one another. Each of the proteins uses different germline encoded Dh and Jh segments to compose the termination of the variable region of the heavy chain. Also, there are additional (N region) nucleotides inserted at the D-J splices of 245.46 antibody and 169.18. Thus, our current structural data support the interpretation that the antisera used for hybridoma selection is specific only for Vh and not an associated Dh, Jh or light chain (from the binding of inulin and GAC antibodies, which bear different light chains).

3. The unusual finding has been made that the CYS-92 residue is absent in the hybridome 245.46 from the CBA /N mouse. This is a striking structural abnormality which causes a failure of intrachain disulfide bridge domain stabilization of the entire V region of this antibody. The defect is accounted for by the unusual joining of the 3' end of the Vh gene to the diversity segment two

codons from the normal termination of the V gene segment. The generality of this defect and its relationship to the overall immune failure of anti-carbohydrate antibody formation are subjects of continuing interest.

Significance to Biomedical Research and the Program of the Institute:

Primary structural analysis of antibodies is the definitive method for classification of families of antibodies. Such basic scientific efforts enhance our understanding of the organization and expression of the immune repertoire of antibodies.

Proposed Course of Research:

To sequence members of the above mentioned families of antibodies and compare this new information with previously established sequence data.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00559-04 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 chrecters 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 Principel Investigator.) (Neme, title, leboratory, and institute affiliation)*

PI:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI
OTHER:	U. Wewer	Visiting Fellow	LP NCI
	C.N. Rao	Visiting Associate	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. The antibodies were also used to screen a cDNA expression library to isolate a putative cDNA clone from the human gene coding for 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.

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

Project Description

Objectives:

a) To isolate and chemically characterize the laminin receptor; b) to produce polyclonal and monoclonal antibodies to the laminin receptor; c) to study the genetics and regulation of the receptor; d) to identify receptors for other matrix components; and e) to study the biosynthesis and regulation of the receptor.

Methods Employed:

Plasma membranes were isolated from cells in log phase of growth or from human breast carcinoma biopsy samples. The plasma membrane homogenate was solubilized in 0.1% Triton X 100, 1.0 - 2.0 mg protein/ml. After centrifugation at 30,000 g for 45 min, the supernate was collected and incubated with SM2 Biobeads (Bio-Rad) to remove the Triton. Iodination of the laminin ligand and the plasma membrane extract was performed using the lactoperoxidase method. Laminin receptors were measured on living cells in suspension. After EDTA removal, the cells were incubated in complete media under constant agitation at 37° for 2 hr. The labeled ligand plus 250 fold excess unlabeled ligand was added and the incubation was continued for 2 hr at 25°C. The cell-bound and free ligand was separated by centrifugation. Binding assays on plasma membrane extracts were performed using one member of the ligand or receptor pair bound to solid phase nitrocellulose Millipore SCWP circles or cyanogen bromide activated Sepharose 4B. In the latter case, 25 µg of laminin or plasma membrane extract protein bound to cyanogen bromide activated Sepharose 4B (100 µl) was mixed with an equal amount of 25 mM Tris, 5 mM $MgCl_2$ and $CaCl_2$, pH 7.4 and 100 µl of this buffer containing 0.1% BSA. ^{125}I-labeled plasma membrane extract (10^8 cpm/mg) or ^{125}I-labeled laminin (10^9 cpm/mg) was added in a total volume of 100 µl, diluted with the buffer. Laminin affinity chromatography was performed using purified laminin cross linked to Sepharose 4B. ^{125}I-labeled plasma membrane extract was incubated 15 hr in the laminin-Sepharose affinity column (1 x 15 cm) at 4°C. The unbound radioactivity was washed with 40 ml of 25 mM Tris, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.9% NaCl, pH 7.4. The bound activity was eluted with 0.2 M glycine HCl, acetic acid, SDS, or urea, pH 3.5, immediately neutralized with 1.0 M Tris saline, and lyophilized. The proteins were identified by slab gel electrophoresis and autoradiography. The number of laminin receptor sites and the Kd were calculated by Scatchard analysis. The laminin receptor isolated from human breast carcinoma tissue was used as an antigen for preparing monoclonal antibodies. Thirteen hybridomas (cloned twice) were obtained which produced IgM antibodies to the receptor as judged by solid phase RIA and immunoblotting.

Major Findings:

Laminin, a glycoprotein of basement membranes which mediates tumor cell attachment

Laminin is a large and complex glycoprotein synthesized by a variety of cell types and deposited in basement membranes. All types of basement membranes (i.e., subepithelial, subendothelial, epidermal, myocyte, etc.) contain

laminin as a major constituent. Laminin serves as a major mechanism for the attachment of metastatic tumor cells to the basement membrane. The vast majority of murine and human tumor cells studied to date utilize laminin as an attachment factor in vitro. Murine tumor cell lines selected on the basis of increased binding affinity for laminin exhibited a greater than ten-fold augmentation in their metastatic propensity in vivo.

By immunoelectron microscopy, laminin is detected throughout the basement membrane, but is present in a higher concentration in the lamina lucida. Laminin as visualized by rotary shadowing electron microscopy has a distinctive cruciform shape with three short arms (35 nm) and one long arm (75 nm). All arms have globular end regions. The short arms may have two globular domains, while the long arm has one end globular domain of a size larger than the globular domains of the short arms. The large size of the laminin molecule is such that a single extended molecule could conceivably span the length of the basement membrane lamina lucida. The specialized structure of the laminin molecule may contribute to its multiple biologic functions. Laminin plays a role in cell attachment, cell spreading, mitogenesis, neurite outgrowth, morphogenesis, and cell movement. Many of these functions may be mediated through specific interaction with the cell surface laminin receptor. Laminin also plays a role in the architecture of the basement membrane by virtue of its ability to bind to multiple matrix components.

By gel electrophoresis, laminin consists of three major types of polypeptide disulfide bonded glycosolated chains: two chains with an approximate molecular weight of 200,000 daltons (B1 and B2 chains), and a large chain of approximately 400,000 daltons (A chain). Translation of mRNA from parietal endoderm cells has provided evidence for two B1 chains migrating as a closely spaced doublet on SDS gel electrophoresis. The polypeptide chains are linked by 50 or 60 disulfide bonds present in the central protease resistant part of the three short arms of the cross. Circular dichroism studies suggest that laminin may contain 20-30% alpha helix and 15% beta sheet structure. Protease digestion studies and Western blotting studies using monoclonal antibodies which recognize the B chains but not the A chains provide evidence that a) at least a portion of the B chains reside in the cross-shaped intersection of the short arms, and b) a portion or all of the A chain is embodied in the long arm.

We have found that the domains of laminin are heterogeneous in function and composition. The carbohydrate structure of the globular end regions of the molecule are different from the rod-shaped regions. For example, the globular end regions but not the rod-shaped regions are rich in exposed α-D-galactosyl end groups. The protease resistant central region of laminin contains the binding site for the laminin receptor presumably on one or more of the rod-shaped regions of the short arms. The long arm of laminin contains a heparin binding site at the globular end region. The long arm also stimulates neurite outgrowth. One or more globular end regions of the short arms promote cell spreading, bind to plasma membrane sulfatides, and also bind to type IV collagen. The type IV collagen binding site for laminin is located approximately 125 nm from the C terminal globular domain.

Laminin receptor identification and characterization

Our laboratory found that many types of normal and neoplastic cells contain high affinity cell surface binding sites (laminin receptors) for laminin. Laminin in solution binds via such specific receptors to suspended or attached cells or to isolated cell membranes. In time course experiments, the plateau of laminin binding is reached after 30 minutes at room temperature. With increasing concentrations of labeled laminin added to the cells or the membranes, the binding is saturable, and occurs with a high ratio of specific to nonspecific binding. Scatchard analysis is linear. The binding affinity constant is in the nanomolar range with 10,000 to 100,000 receptors per cell The human laminin receptor from normal and neoplastic tissue was purified, characterized, and six cyanogen bromide-derived receptor peptides were subjected to microsequence analysis. The receptor bound to laminin-sepharose with an apparent high affinity, allowing the use of washing buffers containing 0.4 M NaCl. A significant fraction of the receptor required SDS or urea for elution. The molecular weight of the receptor from different carcinoma sources and from normal placental tissue was in the range of 68-72,000 daltons. Intrachain but not interchain disulfide bonds influenced the conformation of the receptor as judged by mobility on SDS-PAGE. Isoelectric focusing and 2-D gel electrophoresis indicated the receptor pKa to be 6.0 +/- 0.2, and further, that the protein consisted of one major polypeptide chain. The amino acid composition of the purified receptor exhibited no striking differences among the various tissue sources. By endoglycosidase digestion analysis, the receptor was low in carbohydrate content. Polyclonal antibodies raised against the receptor reacted with an approximately 72,000 dalton component by immunoblotting. There was no evidence of a significant higher molecular weight receptor precursor protein using this method. Peptide mapping with V8 protease yielded a pattern distinct from known serum or matrix proteins of similar molecular weight. Cyanogen bromide generated receptor peptides were purified by reversed phase HPLC. Microsequence analysis of the n terminus region of the cyanogen bromide peptides revealed unique amino acid sequences including (Met-Trp-Trp-Met-Leu-Ala-Arg-Glu-Val-Leu-Arg), (Asp-Glu-Pro-Glu-Pro-Tyr-Ala-Gly) and (Asp-Glu-Pro-Ile-Pro-Tyr) which are not homologous with any known protein sequences by computer-assisted searches. Thus, the laminin receptor is a unique plasma membrane protein with many potential functions.

Monoclonal antibodies to the human laminin receptor recognize different functional domains

In order to study mechanisms regulating the expression and function of the laminin receptor, a library of monoclonal antibodies (mAbs) and polyclonal antibodies were developed in collaboration with Jeff Schlom's lab (DCBD, NIH). The mAbs were prepared against the purified laminin receptor extracted from human breast carcinoma plasma membranes or human placenta. Two mAbs (LR1 and LR2) were found to differ in their effects on laminin binding to the receptor. By solid phase radioimmunoassay, LR1 and LR2 bound with equal titer to the purified receptor. Using immunoblotting, both LR1 and LR2 recognize a single 67 KD component among all the proteins extracted from the membranes of breast carcinoma tissue.

The antibodies also bound with equal titer to isolated microsomal membranes or living breast carcinoma cells. No binding to serum components was evident. When added together with the labeled ligand, mAb LR1 produced a dose dependent inhibition of specific laminin binding to human breast carcinoma cells. In contrast, mAb LR2 had no effect on laminin binding. The two classes of antibodies may therefore recognize different structural domains on the receptor molecule.

Laminin receptor function in tumor cell attachment and metastases

We next studied the effect of these antibodies on the attachment of human melanoma and carcinoma cells to native human amnion basement membrane surfaces. Human amnion membranes consist of a single layer of epithelium, a continuous basement membrane, and an underlying non-vascular interstitial stroma. The epithelium of the amnion can be removed leaving a continuous laminin-rich basement membrane surface. The receptor binding fragment of laminin competitively inhibits attachment of cells to the amnion basement membrane surface. mAb LR1 markedly inhibits attachment of melanoma and carcinoma cells to the basement membrane surface, but not the stromal surface (which lacks laminin). Control antibodies fail to inhibit attachment. Thus, the orientation of laminin in the native basement membrane is such that the cross-shaped receptor binding domain of the short arms is exposed on the attachment surface.

Laminin receptors may be altered in number or degree of occupancy in human carcinomas. This may be the indirect result of defective basement membrane organization in the carcinomas. Breast carcinoma and colon carcinoma tissue contains a higher number of exposed (unoccupied) receptors compared to benign lesions. The laminin receptors of normal epithelium may be polarized at the basal surface and occupied with laminin in the basement membrane. In contrast, the laminin receptors on invading carcinoma cells may be distributed over the entire surface of the cell. They may be unoccupied because of the loss of formed basement membrane associated with the invading cells. Using mAb LR1 we have isolated a putative human cDNA clone for the laminin receptor. The clone was confirmed, in part, by comparing the predicted amino acid sequence with the n terminal sequences obtained from analysis of the CB peptides. This clone will be applied to future studies of the genetic regulation of laminin receptor expression.

The laminin receptor can be shown to play a role in hematogenous metastases. Whole laminin on the tumor cell surface will stimulate hematogenous metastases. This stimulatory effect requires the globular end regions of the molecule (Table 1). Treating the cells with the receptor binding fragment of laminin markedly inhibits or abolishes lung metastases from hematogenously introduced tumor cells. Thus, the laminin receptor can play a role in hematogenous metastases through at least two mechanisms. If the receptor is unoccupied, it can be used by the cell to bind directly to host laminin. If the receptor is occupied with laminin, the cell can utilize the surface laminin as an attachment bridge through the globular end regions. The fragment of laminin which binds to the receptor, but lacks the laminin globular end regions, inhibits both of these mechanisms.

Table 1. Effect of Whole Laminin or its C_1 Fragment on Pulmonary Metastases Formation

Experiment	No. of cells injected	No.	Pulmonary metastases median (range) Control	+ Laminin 10 μg/ml	+ C_1 10 μg/ml
1	0.5×10^5	10	12 (3-22)	72 (38-90)	0
2	0.5×10^5	10	15 (5-18)	65 (33-80)	0
3	0.75×10^5	10	28 (15-30)	110 (45-130)	1 (0-6)
4	0.75×10^5	10	18 (6-26)	80 (30-110)	2 (0-5)
5	1.0×10^5	10	75 (25-90)	165 (80-240)	5 (0-10)
6	1.0×10^5	5	85 (30-100)	122 (24-162)	6 (0-16)
7	2.0×10^5	10	98 (50-106)	210 (150-290)	40 (28-55)

Effect of whole laminin or its C_1 fragment on pulmonary metastases formation. Murine BL6 melanoma cells were trypsinized and allowed to regenerate cell surface laminin receptors. The cells were preincubated for 30 min at 25°C in serum-free media containing whole laminin or the C_1 fragment at a concentration of 10 μg/ml. The washed cells were injected via tail vein. The number of cells injected per mouse and the number of mice (No.) in each group is listed. At 3 wk after injection, all mice were sacrificed and the number of pulmonary metastases were counted in separate lung lobes. Significant differences between the treatment groups and the control ($P<0.05$) were noted for all experiments except no. 7 ($+C_1$ group) using both the Student's t test and the Mann-Whitney test.

Significance to Biomedical Research and the Program of the Institute:

a) A pharmacologic agent which blocks the laminin receptor could be clinically useful in treatment of metastatic disease; b) measurement of laminin receptors in biopsy specimens of human breast cancer could be of diagnostic usefulness.

Proposed Course of Research:

The laminin receptor is the first receptor identified and characterized which binds to a component of the extracellular matrix. It may serve as a prototype for studying the dynamic reciprocity between cells (both normal and neoplastic) and the extracellular matrix environment. Over the next three years, we propose to conduct a series of studies aimed at further elucidating the structure and function of the laminin receptor in normal and neoplastic cells, and attempting to identify new classes of cell surface matrix receptors for other components of the basement membrane.

FUTURE GOALS

1) Evaluate the class of cell membrane transducer systems and the second messenger activated by laminin binding to its receptor. Evaluate the fate of the receptor following binding to soluble or solid phase ligand.

2) Evaluate the hypothesis that more aggressive or highly undifferentiated human carcinomas possess a higher number of unoccupied or abnormally distributed laminin receptors compared to their less aggressive or benign counterparts.

3) Based on predicted protein sequence information derived from the cDNA clone for the laminin receptor, and a cDNA clone coding for the laminin ligand binding domain, determine the amino acid sequence for the laminin ligand binding domain and the receptor binding domain. Use this information to prepare synthetic peptides which competitively block the laminin ligand-receptor interaction. Apply these peptides (or antibodies to the peptides) to experimental metastases imaging and therapy.

4) Attempt to identify, isolate and characterize cell surface receptors for other basement membrane matrix components such as type IV collagen.

Second messenger system activated by the laminin receptor

Laminin binding to its cell receptor illicits a number of biologic responses such as type IV collagenase secretion, mitogenesis and migration. A cytoplasmic pool of laminin receptors exists and polarization of the laminin receptor antigen is related to cell migration and differentiation. The laminin receptor is therefore more than just a simple "anchor" for cell attachment. It is likely that the laminin receptor is similar to other types of receptors in that it operates as a cell membrane transducer with an internal signal carried by a "second messenger".

We propose to study whether the laminin receptor operates through one of the two major known receptor signal pathways. One pathway employs the second messenger cyclic AMP. The other pathway employs a combination of second messengers that includes calcium ions, inositol triphosphate (IP3) and diacylglycerol (DG). Both signal transmission pathways have a common feature: the receptor at the cell surface transmits information through the family of G proteins, which are active if they bind guanosine triphosphate (GTP). The G proteins in turn activate an amplifier enzyme on the inner face of the membrane. The amplifier enzyme influences phosphorylation of precursor proteins which activate internal signals carried by second messengers. In the cyclic AMP pathway the amplifier enzyme is adenylate cyclase, which is regulated by stimulatory and inhibitory G protein pathways.

In the IP3 pathway the amplifier enzymes are phosphodiesterase (PDE) and guanylate cyclase (GC). The second messengers bind to the regulatory component of a protein kinase.

Laminin receptor activation of G proteins common to both types of signal pathways will be examined first. Laminin will be combined with the isolated cell membranes and the stimulation of GTPase activity will be measured. Increase in GTPase activity will implicate the G proteins in either signal pathway. If GTPase stimulation occurs using laminin concentrations which saturate the receptor, then the involvement of the receptor will be confirmed by a) using a series of laminin fragments only some of which contain the receptor binding domain, and b) using the monoclonal or polyclonal antilaminin receptor antibodies to block the G protein stimulation. Treating the membranes with per-

tussis toxin should inhibit the GTPase stimulation. The degree of pertussis toxin inhibition of GTPase has been shown in other systems to be related to the extent of ADP ribosylation of a 41 kDa protein which is characteristic of certain classes of G proteins. Therefore, labeled NAD will be added to membranes to determine the effect of pertussis toxin on ADP ribosylation of a membrane component corresponding to the 41 kDa G proteins in response to laminin binding to its receptor. These experiments should provide general evidence for involvement of G proteins by the activated laminin receptor.

In order to more directly test which signal pathway is activated, experiments will next be conducted using living cells treated with whole laminin or its receptor binding fragment. The time course of changes in intracellular cyclic AMP and IP3 will be directly measured and correlated with the percentage of occupied receptors. Involvement of protein kinases will be studied by examining the phosphorylation of membrane proteins, and cytoskeletal proteins (such as vinculin, vimentin and spectrin), following laminin binding to its receptor. The purified laminin receptor will also be directly tested for protein kinase activity.

Preliminary Data: Recent experimental studies conducted in collaboration with Joel Moss indicate that laminin binding to its receptor in an isolated plasma membrane system can significantly stimulate GTPase activity. Such stimulation could be blocked by pretreatment with the antilaminin receptor monoclonal antibody. Laminin binding to living cells was also shown to increase intracellular cyclic AMP. These preliminary results implicate the adenylate cyclase pathway.

Laminin receptor internalization

Receptors for growth factors or hormones are internalized through a series of specific endocytosis steps involving coated pits. The ligand binds to its receptor which then diffuses into a clathrin coated pit. After a pit buds into the cell and becomes a coated vesicle, the clathrin coat is shed and the vesicle fuses with an endosome. The acidic environment of the endosome causes certain types of ligand-receptor complexes to dissociate. The receptor is subsequently either degraded or recycled to the plasma membrane surface. We plan to investigate whether the laminin receptor is internalized and recycled or degraded. The fate of the laminin receptor may be different from receptors for soluble hormones because laminin is such a large molecule (>800,000 daltons) and because laminin is fixed in the solid phase basement membrane.

Preliminary Data: Soluble labeled laminin binds to suspended carcinoma cells in a time and temperature dependent manner. At 4° the plateau of binding (receptor saturation) is reached after 2 hr. At 20° the plateau is reached within 30 min. At 37° laminin is rapidly bound and internalized. Internalization was verified by protease stripping of the cell surface ligand and immunoelectron microscopy demonstrating laminin antigen within endocytotic vesicles. Internalization of receptors was suggested by immunofluorescent time course studies utilizing antilaminin receptor monoclonal antibodies.

This data will now be extended to include the binding of suspended tumor cells to labeled laminin bound to a solid phase. We will address the following

experimental questions. a) Is the laminin ligand proteolytically modified prior to internalization? b) Is the internalized receptor recycled to the surface or degraded? c) Does the fate of the receptor depend on basal versus apical orientation and the state of the cytoskeletal system?

Relationship of carcinoma receptor expression to clinical aggressiveness

Our previous studies have shown a profound alteration in the distribution and amount of basement membranes during the transition from in situ to invasive human carcinomas of many histologic types. Invasive carcinomas exhibit a marked fragmentation and loss of the basement membrane. The five year survival and incidence of metastases was compared to the degree of differentiation and level of basement membrane loss using antilaminin immunohistology. For colorectal carcinomas and breast carcinomas, the more aggressive more undifferentiated tumors exhibited a statistically significant loss of laminin containing basement membranes. This finding suggests that defective regulation of basement membrane accumulation may accompany the development of the metastatic phenotype. In accord with the loss of formed basement membranes adjacent to the carcinoma cells, there may be an increased expression of both total laminin receptors and unoccupied receptors. We propose to address the following experimental questions: a) For breast carcinomas matched in size and cellularity, is there a significant correlation between the disease stage and the level of occupied or unoccupied laminin receptors? b) Is the level of laminin receptors related to the degree of differentiation, and c) Is the level of estrogen receptors inversely correlated with laminin receptors?

Preliminary Data: A series of 26 cases of human breast carcinoma mastectomy specimens were studied for laminin receptor content. The number of unoccupied receptors per mg membrane protein per DNA content was compared with the number of lymph node metastases. A statistically significant correlation was found between the number of unoccupied receptors and the number of metastases. Immunohistology studies of carcinomas using the monoclonal antibodies raised against the human breast carcinoma laminin receptor revealed an increased antigenicity, and a circumferential rather than basal receptor distribution, in the histologically undifferentiated tumors. Immunohistology using both polyclonal and monoclonal antilaminin receptors have revealed a consistently increased content and cell surface cytoplasmic laminin receptor in undifferentiated endometrial carcinomas compared to premalignant precursor lesions.

This series will now be extended to a total of 200 retrospective breast carcinoma cases, each with a documented clinical course and estrogen receptor determination. Routine measurement of laminin receptors is feasible because samples now used for estrogen receptor determination do not utilize the plasma membrane fraction which contains the laminin receptor.

Amino acid sequence of the ligand and receptor binding domains

Knowledge of the exact amino acid sequences within the binding domain of both the laminin receptor and the laminin ligand itself will provide strategies for designing pharmacologic strategies using synthetic peptides to modify the receptor function. The concept that a short stretch of amino acids can serve as a matrix molecule binding domain has already been established for fibro-

nectin. Rhouslahti's group has shown that the fibronectin cell binding domain is contained in only 4 amino acids: RGDS. This same sequence is not involved in the binding of laminin to cells. We now plan to determine the receptor binding counterpart sequence on laminin. First, the smallest possible proteolytic fragment of laminin will be generated which can competitively inhibit the binding of labeled laminin to whole cells. This will be accomplished using further protease modification of our previously purified laminin fragments. The smallest obtainable laminin receptor-binding fragment will then be analyzed for methionine and cysteine content. If methionine is present in the fragment, it will be further cleaved with cyanogen bromide. If cysteine residues are found, then the binding requirement for disulfide bond associated conformations will be determined using reduction and alkylation. The laminin fragment or its cyanogen bromide peptides will be subjected to microsequence analysis.

The aim will be to obtain the complete amino acid sequence for the receptor binding laminin fragment. If technical reasons (restriction in amount of purified fragment or inability to purify all the CB peptides) prevent the determination of the complete sequence, then the partial sequence will be compared with the predicted sequences from existing cDNA clones (from our lab or outside labs) for laminin. If the fragment coding region cannot be determined from existing clones, then an oligonucleotide probe will be developed and used to obtain a new clone. Alternatively, antibodies to the laminin fragment will be used to screen an expression vector library, to obtain the appropriate laminin cDNA clone. The cDNA clone coding for the laminin binding fragment will be sequenced and the predicted amino acid sequence will then be completed.

Once the complete sequence of the laminin fragment is finally determined, the specific region responsible for binding will be identified. Overlapping sequences of 12 to 20 amino acids will be synthesized which are embodied in the laminin fragment. The synthetic fragments will be purified by reversed phase HPLC and then tested for receptor binding activity using routine binding assays. For the determination of the laminin receptor binding domain sequence, our cDNA clone for the laminin receptor will be utilized. This clone is already known to contain the putative laminin binding domain based on reactivity with the antilaminin receptor mAb which blocks laminin binding to cells. Furthermore, we know that receptor carbohydrate structures are not required for laminin binding. Overlapping synthetic peptides will be prepared corresponding to the predicted sequence of the receptor binding domain for laminin. The synthetic sequences will be screened for laminin binding activity in solid phase assays. If none of the synthetic peptides themselves do not exhibit binding activity, then an immunologic approach will be used. Monoclonal antibodies to the laminin receptor or to the laminin binding fragment will be studied for reactivity with the synthetic peptides. Since some of these monoclonal antibodies block binding of laminin to its receptor and also react with denatured antigen, they are likely to recognize specific sequences near or at the binding domain.

Preliminary data: The previously obtained laminin receptor-binding fragment had a molecular weight of 150,000. (The molecular weight of whole laminin is 900,000). We have now obtained a laminin fragment of 15,000 which retains all of the receptor binding activity and competes 100% for the binding of whole laminin. This fragment is being subjected to microsequence analysis. The labeled laminin fragment was injected into a series of mice bearing experimental

lung metastases. The labeled fragment localized to a statistically significant degree in the lungs of these mice compared to nonmetastases bearing organs or compared to the lungs of control mice.

Identification of tumor receptors for other components of the basement membrane

Receptors for laminin and fibronectin are known to exist. In addition, type I and II interstitial collagens have been shown to bind to the surface of certain types of stromal cells. It therefore seems likely that a family of receptors for matrix proteins exists. The relative expression of each type of receptor may depend on the state of differentiation, environment, or biologic behavior of the given cell. We will therefore attempt to identify and characterize additional cell surface receptors for basement membrane components.

Type IV procollagen will be purified as previously described by our laboratory. Because this large (600,000 Mr) collagen self-assembles into dimers, tetramers and multimers, it is poorly suited for direct binding assays to living cells. Furthermore, laminin on the cell surface will bind to suspended type IV collagen. The choice has therefore been made to go directly to isolation of a type IV collagen binding protein using affinity chromatography. The type IV collagen ligand will be bound to sepharose to produce an affinity matrix. Isolation of binding proteins from cell extracts will be accomplished using methods identical to those successfully applied to the laminin receptor.

Preliminary data: A 100 kDa type IV collagen binding protein has been isolated and purified from human melanoma cells. This protein binds with high avidity to type IV procollagen but not to fibronectin, gelatin, laminin or any other type of collagen. The protein fails to react with polyclonal anti-laminin or antifibronectin antibodies. The 100 kDa protein is not susceptible to collagenase but is destroyed by proteinases.

Over the next year, the 100 kDa protein will be characterized further in terms of binding coefficient, carbohydrate content, amino acid composition, and collagenase activation. In the subsequent year, monoclonal antibodies to the protein will be prepared and studied for their ability to bind to the cell surface and block cell attachment or spreading on type IV collagen substrates. Finally, an attempt will be made to identify the binding domain on the 100 kDa protein and on the collagen ligand itself using approaches described above for the laminin receptor.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00888-03 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Genetic Regulation of Human Type IV Collagenase by Tumor Cells

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

PI:	U.P. Thorgeirsson	Visiting Scientist	LP NCI
OTHER:	M. Ballin	Visiting Fellow	LP NCI
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	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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 standerd unreduced type. Do not exceed the space provided.)*

Type IV collagenase is a basement membrane degrading metalloproteinase that tumor cells secrete to facilitate migration through vascular channels and during the transition from in situ to invasive carcinoma.

Human type IV collagenase was isolated and purified from culture supernatants of A2058 melanoma cells. After ammonium sulfate precipitation and gel filtration chromatography, the final purification of the enzyme was accomplished through type IV collagen affinity chromatography. The type IV collagenase has a molecular weight of 68,000. Amino acid analysis revealed high glycine and serine content. Two dimensional gel electrophoresis of the protein revealed a single polypeptide with an isoelectric point of about 5.8. Polyclonal antibodies were raised in rabbits. After 3-4 months of weekly injections, a low titer of collagenase specific antibody was demonstrated by ELISA.

Co-expression of the metastatic phenotype and type IV collagenase was demonstrated and type IV collagenase was demonstrated in two gene transfer systems, i.e. in H-ras transfected NIH/3T3 cells and in human bronchial epithelial cells that were transformed with v-H-ras through protoplast fusion. These data as well as previous observations showing good correlation between type IV collagenolytic activity and metastatic behavior suggest that this enzyme may represent an important in vitro and in vivo marker for metastatic potential. Preparatory steps have therefore been taken for cloning of the gene for human type IV collagenase using cDNA libraries from a human breast carcinoma or teratocarcinoma cell lines that express type IV collagenase.

Project Description

Objectives:

1. To purify sufficient amounts of type IV collagenase from human tumor cell culture supernatants for raising of a) polyclonal antibodies, b) monoclonal antibodies, c) and doing protein sequencing.

2. To isolate a cDNA clone of human type IV collagenase using either collagenase antibody or an oligonucleotide probe derived from the protein sequence information of type IV collagenase.

3. Study transcriptional regulation of type IV collagenase in malignant cells.

Methods Employed:

1. Collection of serum-free culture supernatants of A2058 melanoma cells.

2. Gel filtration chromatography using Bio Gel 0.5 m as a purification step for type IV collagenase.

3. Extraction and purification of type IV collagen from EHS tumors and making type IV affinity column for final purification of type IV collagenase.

4. Immunization of rabbits with the purified type IV collagenase in complete Freund's adjuvant.

5. Testing for type IV collagenase antibody and specificity by ELISA.

6. Two dimensional gel electrophoresis of the purified enzyme.

7. Microsequencing of the purified protein and development of a synthetic oligonucleotide probe.

Major Findings:

Type IV collagenolytic activity can be turned on through gene transfer technology using either genomic DNA from malignant cells or cloned Harvey ras oncogenes. This was demonstrated in NIH/3T3 cells transfected with DNA from leukemic cells, T24 human bladder carcinoma, isolated c-H-ras from T24 cells, or viral H-ras. The NIH/3T3 cells had minimal type IV collagenolytic activity, which was increased up to 700% in the c-H-ras transfectants that were able to form both experimental and spontaneous metastases in nude mice. A similar observation was made using primary human bronchial epithelial cells as recipient cells for the v-H-ras. These ras transformants that were metastatic in nude mice expressed up to 500% increase in type IV collagenolytic activity.

Type IV collagenase was isolated from A2058 melanoma cells. The purified enzyme has a molecular weight of 68,000. Two dimensional gel electrophoresis demonstrated a single polypeptide with an isoelectric point of about 5.8.

Polyclonal antibodies were raised in rabbits. After 3-4 m of immunizations with the purified enzyme, a low titer of type IV collagenase specific antibody was demonstrated by ELISA.

Significance to Biomedical Research and the Program of the Institute:

Type IV collagenase specifically cleaves basement membrane collagen and is produced and secreted by malignant cells to facilitate vascular invasion. This enzyme may serve as one of the most important biochemical markers for metastatic potential of malignant cells. It is, therefore, of utmost importance to understand how it is regulated during the metastatic process. Isolation of a cDNA clone for the gene for human type IV collagenase will enable us to study its transcriptional regulation in malignant cells.

Proposed Course of Research:

1. Protein sequencing of type IV collagenase, either directly or sequencing HPLC isolated fragments produced by cyanogen bromide or proteolytic enzymes. The sequence information will be used for synthesis of an oligonucleotide probe that will then be used to screen a cDNA library.

2. Acquisition of human tumor cell cDNA libraries. At present, teratocarcinoma and mammary carcinoma libraries are available. If necessary, a cDNA library will be made from cells that have very high type IV collagenolytic activities. The libraries will be screened with a polyclonal type IV collagenase antibody or an oligonucleotide probe, as mentioned earlier. Positive plaques will be subcloned into appropriate plasmids for restriction enzyme analysis and DNA sequence.

3. Expression of type IV collagenase in human tumors will be studied. This will be done by Northern blot hybridization of tumor RNA with ^{32}P-labeled type IV collagenase cDNA clone. In situ hybridization technique will also be used to quantitate its expression by individual cells within the same tumor.

Publications:

Turpeenniemi-Hujanen, T., Thorgeirsson, U.P., Hart, I.R., Grant, S.S., and Liotta, L.A.: Expression of collagenase IV (basement membrane collagenase) activity in murine tumor cell hybrids which differ in metastatic potential. J. Natl. Cancer Inst. 74: 99-103, 1985.

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., and Liotta, L.A.: Mechanisms of tumor invasion and their potential therapeutic modifications. In Mihich, E. (Ed.): Biological Responses in Cancer, Vol. 4. New York, Plenum Publ. Corp., 1985, pp. 21-46.

Turpeenniemi-Hujanen, T.M., Thorgeirsson, U.P., Rao, C.N., and Liotta, L.A.: Laminin increases the release of type IV collagenase from malignant cells. J. Biol. Chem. 261: 1883-1889, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00889-03 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Laminin and Laminin Receptor in Tumor Progression

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

PI:	U. Wewer	Visiting Fellow	LP NCI
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	I.M.K. Margulies	Biologist	LP NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	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 standerd unreduced type. Do not exceed the space provided.)*

A new tissue model has been developed to study the progression from normal to metastatic adenocarcinoma of the endometrium. The interstitial matrix around the stromal cells of the proliferative phase of the normal menstrual cycle was unreactive with antibodies to laminin. However, commencing with the secretory phase, stromal cells accumulated distinct cytoplasmic- and pericellular immunoreactive material. The maximal amount of stromal cell associated laminin was observed in predecidual cells of the late secretory phase. Thus, laminin immunostaining discriminates stromal cells of the proliferative phase (being "negative") from those in the secretory phase (being "positive"). Sixty-six cases of endometrial hyperplasia and adenocarcinomas were also stained with antibodies to laminin. Sixty-nine percent of biopsies of cystic hyperplasia and thirty percent of adenomatous hyperplasia contained laminin positive stromal cells. In contrast, stromal cells in the atypical adenomatous hyperplasia and adenocarcinomas did not react with antibody to laminin. The expression of laminin receptor in the stromal cells codistributed with laminin. Adenocarcinoma cells exhibited markedly enhanced quantities of laminin receptor compared to normal endometrial glands which contained polarized basal laminin receptors. Basement membranes of the surface epithelium, the glandular epithelium and of the vessels stained strongly with antibodies to laminin. In pre- and neoplastic tissues, laminin immunostaining revealed discontinuous and defective basement membranes. In poorly differentiated carcinomas only sparse amounts of laminin positive basement membranes were observed; these tumors in contrast exhibited cytoplasmic laminin and also significant immunoreaction with antibodies to laminin receptor. Taken together, these data point to a dynamic interrelationship between the endometrial glands and the stromal cells reflected in the extracellular matrix. This interrelationship may be disrupted during the progression to invasive adenocarcinoma.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

1. Develop a tissue model to study the role of the laminin receptor interaction during the progression from normal epithelium to metastatic carcinoma.
2. Prepare large quantities of homogeneously pure human laminin receptor.
3. Biochemically characterize the receptor itself and the fate of the receptor ligand complex.

Methods Employed:

Tissues

Specimens of human endometrial tissue were obtained by curretage or hysterectomy during routine diagnostic or therapeutic procedures conducted in Copenhagen, Denmark. A total number of 144 cases were included. a) Biopsies from 44 women aged between 17-53 years were investigated; 15 were in the proliferative phase (day 4-14), 9 in the early secretory phase (day 14-17), and 20 in the late secretory phase (day 17-28). Histological dating was performed according to the criteria of Noyes. b) 4 women (aged 30-40 years) had received gestagen (Orthonett Novum, 0.5 mg norethisteron, 35 μg ethinyleostradiol) for 2-10 years prior to curretage. c) In 52 patients endometrial biopsies revealed hyperplastic lesions. The hyperplasia was classified according to WHO using morphologic criteria as detailed by Kurman et al. into three groups; 29 cases of cystic (proliferative, simplex) hyperplasia (aged 31-66 years, mean 49.1 years), 10 cases of adenomatous hyperplasia (aged 42-62 years, mean 50.3 years), and 13 cases of atypical adenomatous hyperplasia (aged 42-70 years, mean 60.7 years). d) In 14 cases (aged 55-72 years, mean 63 years) endometrial adenocarcinoma was diagnosed. The carcinomas were classified according to WHO into well-differentiated, moderately and poorly differentiated adenocarcinomas.

Immunoperoxidase Staining

Immunostaining was carried out as previously described. Most of the tissues were fixed in neutral buffered formalin or in ethanol:acetic acid. Pretreatment of sections with pepsin (5 mg/ml, Sigma, St. Louis, MO) or trypsin (0.5 mg/ml, Sigma) was used. Polyclonal rabbit antiserum against rat laminin purified from the L2 yolk sac carcinoma was produced and characterized as described. A dilution of 1:500 of the antiserum was used. Affinity purified anti-laminin antibodies were used at a concentration of 1-5 μg/ml. Monoclonal antibody (4E10) against human laminin was used in a dilution of 1:1000 of ascites fluid or 10 μg/ml of purified IgG_1. Rabbit anti-human laminin receptor antiserum was used at a dilution of 1:100. The antibodies were diluted in 50 mM Tris-HCl, pH 7.4 and incubated with the sections for 2 hrs at room temperature. The secondary antisera, swine anti-rabbit IgG and peroxidase anti-peroxidase rabbit serum were purchased from Dakopatt (a/s, Copenhagen, Denmark) and used in a dilution 1:50. The peroxidase reaction was visualized by 5 mg/10 ml of 3.3' diaminobenzidine in 50 mM Tris-HCl, pH 7.2, and 0.03% H_2O_2. The washing buffer was 0.25 M NaCl in 50 mM Tris-HCl, pH 7.2. In control sections the specific antisera were replaced by absorbed antisera or nonimmunized rabbit serum.

Electron Microscopy

Endometrial biopsies from 6 cases of cystic hyperplasia were examined by electron microscopy. The specimens were processed as follows: Tissue blocks were promptly fixed at room temperature in Karnovsky fixative (2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2) for 1 hr. All specimens were treated with 1% tannic acid (Mallinckrodt, Inc., St. Louis, MO) as described. The samples were dehydrated in graded ethanols, postfixed in 2% osmium-tetroxide, and embedded in Epon. Ultrathin sections from selected areas were collected upon copper grids, stained with uranyl acetate and lead citrate and examined with a Jeol 500 electron microscope.

Major Findings:

Laminin in the Stromal Cells of the Normal Human Endometrium

In the proliferative phase, the stromal compartment of the endometrium consisted predominantly of small spindle-shaped cells. Immunostaining with anti-laminin antibodies failed to identify detectable amounts of laminin associated with these cells, either within the cytoplasm or in the pericellular matrix. In this respect, the endometrial stromal cells of the proliferative phase did not differ from fibroblast-like stromal cells in other tissues.

With the onset of the secretory phase, the stromal cells of the endometrium gradually undergo major morphological changes, which is apparent at day 24 and is termed predecidualization. Predecidualization was accompanied by the appearance of intracellular and pericellular laminin. At day 14-17, before histologically recognizable predecidualization could be identified, scattered immunoreactive laminin was seen in the cytoplasm of stromal cells close to the small arteries and underneath the endometrial surface.

The number of stromal cells exhibiting laminin positive immunostaining as well as the intensity of the staining reaction gradually increased throughout the secretory phase. In late secretory phase, most (>90%) predecidualized stromal cells were positive. The positive immunostaining was found in the cytoplasm and also discontinuously in the pericellular matrix. Stromal cells located in the basal part of the endometrial biopsies consistently appeared unreactive. All controls using preimmune or absorbed sera were also negative. In a given section with abundant stromal cells the extent of extracellular laminin-positive material varied from scattered punctate deposits on the cell surface to a "semicircular" rim of immunoreactive material surrounding portion of individual cells. In no cases did we observe a continuous circumferential staining pattern typical of fully decidualized stromal cells of pregnancy.

In four cases investigated, women had been treated for prolonged periods of time with gestagen. This treatment is known to induce the formation of predecidualized stromal cells indistinguishable from decidual cells of pregnancy. In several areas of these four endometrial biopsies, stromal cells were surrounded by a continuous pericellular sheath of laminin.

Laminin in Stromal Cells of Endometrial Hyperplasia and Endometrial Carcinomas

Immunohistochemical localization of laminin was studied in 52 cases of endometrial hyperplasia including 29 cases of cystic hyperplasia, 10 cases of adenomatous hyperplasia, and 13 cases of atypical adenomatous hyperplasia. Furthermore, 14 adenocarcinomas were investigated. All types of hyperplasia contained densely packed, spindle-shaped stromal cells indistinguishable morphologically from stromal cells of the proliferative phase. However, immunohistochemical staining for laminin revealed a distinct difference among these three types of hyperplasia groups. In 69% of the cases of cystic hyperplasia laminin positive material was seen both in the cytoplasm of stromal cells and in the form of small extracellular aggregates. Thus, with antibody to laminin one could identify a population of stromal cells, not otherwise appreciated on hematoxylin-eosin stained sections. Only 30% of the adenomatous and none (0%) of the atypical adenomatous hyperplasia demonstrated laminin immunoreactive material. All 14 cases of adenocarcinomas examined were devoid of laminin positive stromal cells.

In an effort to further correlate the localization of the laminin positive material with a subcellular structure at the ultrastructural level, we performed electron microscopy on 6 cases of endometrial biopsies characterized by cystic hyperplasia. At the ultrastructural level, stromal cells in hyperplastic endometrium resembled fibroblasts. In the nuclei, euchromatin predominated and several nucleoli were recognized. In the cytoplasm, distended rough endoplasmic reticulum contained granular material. Collagen fibers were seen extracellularly close to the cell surface. Some stromal cells retained granular basement membrane-like material confined to the cell surfaces. In some areas, we were able to identify fragments of what appeared to be a typical basement membrane with a lamina densa and rara-like structure.

Distribution of Laminin in Epithelial Basement Membranes

Epithelial basement membranes of the endometrial glands, vessels and smooth muscles were positive for laminin as expected. In cystic and adenomatous hyperplasia the epithelial basement membranes could be visualized by laminin staining as a continuous band around the glands. In atypical adenomatous hyperplasia the staining of basement membranes was often discontinuous. Localization of laminin in the carcinomas of the endometrium revealed that several tumor elements especially in the well- or moderately differentiated type of carcinomas possessed basement membrane-like structures, but the staining was in a discontinuous pattern. In the poorly differentiated carcinomas, only focal remnants of basement membranes. Cytoplasmic staining for laminin was found in several of the adenocarcinoma cases but most intensely in poorly differentiated tumors.

Laminin Receptor in the Human Endometrium

In normal endometrial glands, immunoreactive material was confined to the basal region of the glands and near the zone of the basement membrane. In the proliferative phase the endometrial stromal cells were negative, while in secretory phase they were slighly positive for the laminin receptor. As the stromal cells gradually changed to predecidual cells, an increasing

laminin receptor staining was found. In the hyperplasia, several glands showed more diffuse cytoplasmic staining. This type of staining reaction was markedly increased in the carcinomas especially when poorly differentiated tumor elements were present.

Significance to Biomedical Research and the Program of the Institute:

Adenomatous and atypical adenomatous hyperplasia, without adequate therapy, may progress to adenocarcinoma of the uterus. In the past, studies on the biology and grading of hyperplasia have not generally employed immunohistochemistry. With this approach, we demonstrated that stromal cells in 69% of the cases classified as endometrial cystic hyperplasia exhibited laminin immunoreactive material. On the other hand, adenomatous hyperplasia, considered to be a more ominous form of hyperplasia, had significantly less laminin in the stroma. In the cases of atypical adenomatous hyperplasia and in adenocarcinomas, no laminin positive stromal cells were identified. These results suggest that the progression from cystic to atypical adenomatous hyperplasia and overt carcinoma is associated with decreasing amounts of stromal laminin and increasing amounts of laminin receptor. These findings are of direct diagnostic significance.

Proposed Course of Research:

1. Use the cDNA clones for laminin and its receptor to study the progression from normal endometrium to adenocarcinoma using a) in situ hybridization, b) Northern hybridization, c) Southern hybridization.
2. Extend these studies to tumor progression in other tissues.

Publications:

Horan Hand, P., Thor, A., Rao, C.N., Schlom, J., and Liotta, L.A.: Expression of laminin receptor in normal and carcinomatous human tissues as defined by a monoclonal antibody. Cancer Res. 45: 2713-2719, 1985.

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Bryant, G., Haberern, C., Rao, C.N., and Liotta, L.A.: Butyrate induced reduction of tumor cell laminin receptors. Cancer Res. 46: 807-811, 1986.

Togo, S., Wewer, U.M., Margulies, I.M.K., Rao, C.N., and Liotta, L.A.: Monoclonal antibodies to the human laminin receptor inhibit cell attachment to native human basement membranes. In Shibata, S. (Ed.): Basement Membranes. New York, Elsevier Science Publ., 1985, pp. 325-332.

Liotta, L.A.: Molecular biology of metastases: A review of recent approaches. Eur. J. Cancer Clin. Oncol. 22: 345-348, 1986.

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Wewer, U.M., Damjanov, A., Weiss, J., Liotta, L.A., and Damjanov, I.: Mouse endometrial stromal cells produce basement membrane components. Differentiatio (in press)

Wewer, U.M., Liotta, L.A., Jaye, M., Ricca, G.A., Drohan, W.N., Claysmith, A.P., Rao, C.N., Wirth, P., Coligan, J.E., Albrechtsen, R., Mudryj, M., and Sobel, M.E.: Altered levels of laminin receptor mRNA in human carcinoma cells with varying ability to bind laminin. 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 00891-03 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Chemotaxis in Tumor Cells

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

PI:	E. Schiffmann	Research Chemist	LP NCI
OTHER:	M. Stracke	Biotechnology Fellow	LP NCI
	R. Guirguis	Guest Researcher	LP NCI
	R. Muschel	Sr. Staff Fellow	LP NCI
	L. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

Robert Bassin, Chief, Cellular and Molecular Physiology Section, LTIB, NCI; Joel Moss, LCM, NHLBI; B.F. Sloane, Department of Pharmacology, Wayne State Univ., Detroit, Michigan

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.67	2.67	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are studying biochemical events characteristic of malignant tumor cells, which must be highly motile while invading tissue and metastasizing to distant sites. We have found that a number of metastatic cell lines produce and respond to autocrine motility factors. A partially purified material from the conditioned media of a human melanoma cell line was found to be a protein with an Mr of about ~ 55 KD. The material induces a strong chemotactic response in the producer cells and appears to exert its action by perturbing membrane phospholipid metabolism of the cell. Further studies on the mechanism of action of the motility factor indicate that Gi proteins are involved in the amplification of the motility signal while the Gs proteins may be involved in modulation of the signal. Other investigations on factors affecting regulation of motility indicated that cathepsin B in the cell membrane may play a role in both the adherence and migration of melanoma cells. In studies on the occurrence of motility factors in tumor cells, we have found that a variety of transformed cells including both metastatic and non-metastatic produce and respond to motility factors. In addition, these cells as well as some revertant lines migrate to the melanoma factor. We have also developed further purification procedures in producing melanoma motility factor. These will assist us in the preparation of sufficient material to clone the gene for the factor and subsequent studies on its role in metastasis.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To study the biochemical aspects of chemotactic motility in tumor cells and to determine whether such motility is correlated with the metastatic potential of various tumor cells.

Methods Employed:

The modified Boyden chamber filter assay for chemotaxis was used. Both high performance liquid chromatography and ordinary gel filtration procedures were employed to isolate active material from conditioned media.

Major Findings:

Studies on the autocrine motility factor (AMF) previously found to be produced by and to stimulate human melanoma cell migration have proceeded in a number of areas:

a) large scale preparation of purified material

Alternate exposure of cells in culture to serum-containing and serum-free media has resulted in a 10-fold increase in the activity of AMF in the serum-free media. This has enabled us to institute large scale production of purified AMF. The serum-free conditioned media is dialyzed against buffer and applied to a high-performance liquid chromatography (HPLC) anion exchange column. Biologically active fractions were analyzed by gel electrophoretic procedures and found to contain about 5 components. The active fractions were applied to molecular sieve chromatography and the active fractions contained a reduced number of proteins. This active material was then subjected to cation HPLC, resulting in further purification. A single active 55 kDa component has been purified and isolated. It exhibits activity at the NM level.

b) mechanism of action and modulation of AMF

We have found that pertussis toxin added to cells even 1 hr after the state of a chemotaxis assay caused significant inhibition of motility (45%). Toxin added earlier resulted in virtually complete inhibition, strongly suggesting that a Gi protein may be involved in transducing the chemical signal of AMF interacting with its putative cell receptor. Additionally, toxin depressed spontaneous motility of melanoma cells. Collaborative results with J. Moss and coworkers indicated that AMF stimulated GTPase in cell membrane fractions, a result consistent with the involvement of G proteins. In whole cell fragments, stimulators of cyclic AMP such as cholera toxin and forskolin were not as effective as pertussis toxin in inhibiting cell motility, suggesting that activated Gs proteins might play a modulatory but not direct role in the AMF stimulated motility. The effect of 8-bromocyclic AMP confirmed this.

Studies using another approach to the regulation of cell motility were performed with the collaboration of Dr. B.F. Sloane of Wayne State University. We found that 0.1 μM of a cathepsin B inhibitor eliminated motility without toxicity

to cells. Higher levels inhibited adherence of the cells. Cathepsin B location on membranes of certain mouse melanoma have been correlated with enhanced metastatic capacity. It is conceivable that this enzyme may function as an attachment factor for the cell to a biological matrix or be involved in contractile events of cell spreading during attachment and migration.

c) other transformed cells with AMF activity

We have found in further studies on the prevalence of AMF in transformed cells that both nonmetastatic (C127, ras H, V-sarc, e.g.) and metastatic (C127, ras H, V-sarc) varieties produce and respond to motility factors that they produce. 3T3 cells do not elaborate a motility factor for themselves. In addition, some revertants of transformed cells do not produce motility factors for themselves. Furthermore, some revertants as well as 3T3 cells, although they do not produce motility factors for themselves, do respond to AMF from melanoma cells. These results suggest that part of the transformed phenotype include both the ability to produce AMF and the capacity to respond to it.

Significance to Biomedical Research and the Program of the Institute:

A primary feature of tumor cell metastasis is the ability of the cells to locate at target sites distant from the original tumor mass. This requires that these cells, if they spread via the vascular route, e.g., penetrate the endothelium of the vascular system, enter the blood flow, and again penetrate the endothelium from the luminal side to migrate to another target site. At least two processes are involved in traversing basement membrane of the endothelium -- digestion of membrane components and chemotactic migration of the cells. Little is known about either the chemoattractants or the biochemical events that contribute to tumor cell motility. If unique attractants are identified that stimulate the migration of these cells from either the extravascular or luminal sides of the vasculature, it well may be possible to design antagonists that would inhibit migration or develop antibodies that form complexes with the attractant with a similar result. Our results with the conditioned medium attractant may provide a basis to pursue this strategy. Similarly, if the molecular events within the cell are clarified, it may be possible to interfere with some of the post-receptor processes in the motile response. Additionally, we may be able to identify with confidence at least two properties of the transformed state: the ability of a tumor cell to produce its own motility factor and a receptor on the cell enabling it to respond to the factor. These properties, like autocrine growth factors, would give the tumor cell a relative degree of independence of the host.

Proposed Course of Research:

We will obtain large amounts of the homogeneous protein, and will analyze it for its component amino acids and other residues, if any, such as carbohydrates and covalently modified amino acids. We would then attempt sequence determination of the protein and selective enzymatic cleavage to identify the active site. We would then want to determine whether the material is related to known oncogene products. We will prepare large scale amounts of AMF for the production of an antibody to it. Achieving this will serve at least two purposes: cloning the gene for AMF and screening a variety of conditioned media from

tumors for AMFs with common determinants. For cloning the genes, either the antibody or synthetic oligonucleotide probes based upon partial sequence information of AMF will be useful in screening cDNA or genomic DNA libraries, obtained by molecular biological techniques. We will also subject purified AMF to limited proteolytic digestion in order to isoalte active fragments and subsequently to identify the active site of AMF. We would label AMF to identify a cell surface receptor and we will pursue studies of post-receptor events by investigating the possible role of G proteins, phospholipases A and C_1 and calcium flux in the transduction process. We will also further study the role of cathepsin B in both the adherence and motility of melanoma cells.

Publications:

Terranova, V.P., DiFlorio, R., Hujanen, E.S., Lyall, R.M., Liotta, L.A., Thorgeirsson, U.P., Siegal, G.P., and Schiffmann, E.: Laminin promotes neutrophil motility and attachment. J. Clin. Invest. 77: 1180-1186, 1986.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00892-03 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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:	M.E. Sobel	Senior Investigator	LP NCI
	G. Bevilacqua	Guest Researcher	LP NCI
	A.P. Claysmith	Biologist	LP NCI
	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	2.0	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 molecular biology of tumor metastasis has been investigated. A model system consisting of seven related murine K1735 melanoma cell lines which vary in metastatic potential was used for study. A cDNA probe, pNM23, recognizes two RNA species which are expressed to a greater extent in RNA of low metastatic K1735 cell lines than in related higher metastatic K1735 cell lines. The restriction map and DNA sequence of this cDNA were obtained. Computer analysis of the predicted amino acid sequence of the only open reading frame indicates that the NM23 gene encodes a novel protein. In contrast, a cDNA probe to the basement membrane protein type IV collagen recognizes RNAs more abundant in highly metastatic K1735 cell lines than in related, low metastatic cell lines. Synthesis and secretion of type IV collagen and other basement membrane components, laminin and entactin, are relatively increased in highly metastatic K1735 cell lines. Cellular analysis of K1735 melanoma metastatic potential determined that potential to invade basement membranes and factors involved in colonization are critical to observed differences in K1735 melanoma metastatic potential; cellular sensitivity to host immune responses were not a determining factor. Metastases in this model system can therefore be associated with changes in activity of specific genes. Planned experiments will determine the diagnostic and therapeutic potential of these cDNA probes.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

The objective of this project is to determine those genetic elements whose expression distinguishes neoplastic cell behavior from the phenotype of benign tumor cells and normal tissue. In the first year of this project, a variety of cDNA probes were tested for their relative expression in RNAs from a series of related K1735 murine melanoma cell lines that differ in metastatic potential. Several of these cDNA probes which appeared to distinguish metastatic potential in the K1735 murine melanoma model system have been further analyzed. Our immediate goals are to:

1. Determine the correlation of relative RNA levels corresponding to the cDNA probes and metastatic potential in the K1735 melanoma model system.

2. Investigate the relative expression of the gene products of the cDNA probes by the K1735 melanoma cell lines.

3. Investigate the cell biology of K1735 metastasis, to determine what specific metastatic cell functions are associated with altered gene expression.

Methods Employed:

Molecular analysis of metastasis has utilized a series of related murine melanoma (K1735) cell lines which differ in metastatic potential. The original K1735 lines were developed elsewhere and extended by members of the TIM section in collaboration with others. Relative levels of RNAs produced by each K1735 melanoma cell line corresponding to cDNA probes were assessed by Northern Blot hybridization. The pNM23 cDNA probe was analyzed by restriction endonuclease digestion and its DNA sequence was determined using the chemical methods of Maxam and Gilbert.

The metastatic potentials of each K1735 melanoma cell line were determined by i.v. and s.c. injections into C3H/HeN and NIH nude mice. Cells of each K1735 cell line were radiolabeled for in vitro analysis of their sensitivity to activated macrophage and natural killer cell lysis, and their potential to invade human amnion basement membranes.

Major Findings:

1. Gene Activities Associated with Low Metastatic Potential

In the first year of study, the pNM23 cDNA clone was identified by differential hybridization of mRNA from low and highly metastatic K1735 melanoma cell lines. This cDNA recognizes two bands of RNA on Northern blots, present to a greater extent in low metastatic Clone 16 and Clone 19 K1735 RNAs than in the RNA of five related, highly metastatic K1735 cell lines (M2, M4, TK, TK-Eve, TK-Liver). A second series of RNAs were extracted from each K1735 cell line, in which in vitro parameters such as passage number and confluency were varied. The RNAs were used to confirm the inverse correlation between metastatic potential and RNA levels corresponding to the pNM23 cDNA clone. The relationship of

NM23 gene expression to metastatic potential in vivo was investigated. The pNM23 cDNA probe exhibited significantly greater hybridization to RNAs extracted from subcutaneous primary tumors formed by low metastatic Clones 16 and 19 than to primary tumors formed by the related, highly metastatic cell lines. The pNM23 cDNA insert hybridized to oligo (dT) selected K1735 Clone 19 RNA, and presumably encodes a mRNA. The cDNA probe also recognizes RNA bands on Northern blots from murine NIH 3T3 and F9 teratocarcinoma cells and human melanoma cell lines. Restriction mapping of the pNM23 cDNA was performed, and its DNA sequence was determined. Analysis of the DNA sequence reveals only one open reading frame. The predicted amino acid sequence of the open reading frame was analyzed by the Fast Protein Data Base, and is a novel sequence. Metastasis in this model system is therefore accompanied by alterations in the levels of specific RNAs. Further, the progression of cells toward a more highly metastatic behavior may not involve just the acquisition of invasive and other traits, but may also involve a relative decrease in the expression of certain genes.

2. Gene Activities Associated with High Metastatic Potential

The synthesis and expression of basement membrane components by the K1735 murine melanoma cell lines was studied. RNAs extracted from highly metastatic K1735 cell lines (M2, M4, TK, TK-Eve, TK-Liver) contained significantly greater hybridizable amounts of type IV collagen message than did the related, low metastatic K1735 cell lines (Clone 16, Clone 19). Immunoprecipitation of biosynthetically labeled type IV collagen from each K1735 melanoma cell line revealed the same direct correlation of type IV collagen synthesis and metastatic potential. In addition, relative levels of biosynthetically labeled basement membrane components laminin and entactin were increased in the more highly metastatic melanoma cell lines. Thus, the production of basement membrane proteins is associated with the development of the metastatic phenotype in this model system.

3. Cellular Analysis of K1735 Melanoma Metastasis

Metastasis involves a myriad of cell functions including detachment from the primary tumor, intravasation and extravasation of the immune system, colonization of a distant organ and avoidance of host immune responses. The K1735 melanoma model system used for our molecular analysis has been studied for several component cell functions in metastasis, in order to more precisely define the nature of the differences in metastatic potential under investigation. The relative susceptibility of the K1735 melanoma cell lines to T-lymphocyte, natural killer cell and activated macrophage cytotoxic responses were not significantly different, suggesting that differences in K1735 melanoma metastatic potential are not the result of different sensitivities to host immunological responses.

Invasion of basement membranes is thought to be critical to the intravasation and extravasation of the circulatory system by metastasizing cells. Clone 19, one of two low metastatic K1735 cell lines studied, exhibited relatively reduced ability to degrade and transverse human amnion basement membranes in vitro. Invasive capability may therefore contribute to different metastatic

potentials in K1735, but the pattern of invasive capability among the K1735 melanoma lines is not consistent with differences in RNA levels corresponding to the NM23 or basement membrane protein genes.

The size of primary tumors formed at the site of subcutaneous injection by the K1735 melanoma cell lines varies with their metastataic potential and RNA levels corresponding to NM23 and type IV collagen genes. Kinetic studies, in which primary tumors formed by each K1735 line were measured weekly, indicate that differences in primary tumor formation are principally the result of differing lag times before the onset of tumor growth, and that tumor growth rates were not significantly different. This observation is supported by equivalent growth rates in vitro of the K1735 melanoma cell lines. The development of relatively smaller primary tumors by low metastatic Clone 16 and Clone 19 cell lines does not directly cause their low metastatic rate. This was demonstrated by extending the time post-injection for low metastatic cell lines to permit the development of primary tumors equivalent in size to those of the higher metastatic cell lines at shorter times post-injection. In these experiments, the low metastatic phenotype was maintained. We hypothesize that factor(s) involved in colonization (i.e., angiogenic capability, production of a suitable microenvironment for growth) contribute to differences in both metastatic capability and primary tumor formation in this model system.

Significance to Biomedical Research and the Program of the Institute:

Tumor metastasis remains the major cause of treatment failure for patients with solid tumors. Our experiments indicate that in a metastasis model system, metastatic potential is accompanied by changes in the activity of specific genes. This work provides a novel approach to understanding the biology of metastasizing tumor cells. Planned experiments, described below, will investigate the relevance of these cDNA probes to metastasis in a variety of diseases, and their molecular regulation. Such experiments may point to both diagnostic and therapeutic advances.

Proposed Course of Research:

The NM23 and basement membrane protein genes will be tested by Northern blot and in situ hybridizations, to determine their relative expression in other metastasis model systems and human surgical specimens. Such studies will address the questions of the relevance of these cDNA probes to metastasis as it occurs in numerous cancers and possible heterogeneity of expression among multiple metastases.

Based on these studies, full-length genomic and cDNA clones may be obtained, including regulatory regions. These clones can then be used in transfection studies, to examine the effect of gene expression on metastatic potential, and for investigations of the molecular regulation of these genes.

The predicted amino acid sequence of the NM23 gene will be used to produce synthetic peptides, to which antibodies can be generated. These antibodies will be instrumental in determining the nature of the NM23 gene product and what aspects of the metastatic process it is involved in.

Molecular hybridization of cDNA and mRNA from paired high and low metastatic cell lines is being used to identify additional cDNA probes which discriminate metastatic potential. The cloning of multiple metastasis-associated cDNA probes increases our chances of identifying a gene(s) which can lead to diagnostic and therapeutic advances in the treatment of metastatic disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00893-03 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

DNA Mediated Transfer of Metastatic Potential

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

PI:	R.J. Muschel	Senior Staff Fellow	LP NCI
OTHER:	K. Gomi	Visiting Scientist	LP NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI
	E.W. Chu	Chief, Cytopathology Section	LP NCI

COOPERATING UNITS *(if any)*

R. Pozzatti (Fellow) and G. Khoury (Chief), LMV, NCI

LAB/BRANCH

Laboratory of Pathology

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We had previously observed that transformation by ras^H of NIH-3T3 cells resulted in metastatic as well as tumorigenic cells. Since cells transformed by v-sarc or v-mos were not metastatic, but were highly tumorigenic, the property of inducing metastasis appeared to be unique to the ras^H gene (Muschel et al., Am. J. Pathol. 121: 1-8, 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.

With E. Chu and K. Nakahara, we examined the karyotypes of these cells. Surprisingly, the ras^H transformed rat embryo cells had either remained diploid or near diploid. Even in the metastases in nude mice derived from these cells, the karyotype was unchanged from the parental cell line. This suggests that karyotypic selection does not play a role in the metastatic process for these cells.

However, ras^H did not induce metastatic potential in all cell lines tested. For example, C127 cells, a murine epithelioid line, did not metastasize after transformation by ras^H although the cells were highly tumorigenic. Metastatic potential has been induced in these cells by gene transfer and attempts are under way to clone the gene involved.

Project Description

Objectives:

1. We have been able to transfer the metastatic potential to the nonmetastatic, tumorigenic ras^H transformed C127 using DNA transfer. Our goal is to clone the gene involved.

2. We have shown that ras^H transformed rat embryo fibroblasts are diploid or near diploid as are ras^H plus myc transformants. We intend to develop techniques for in situ hybridization to allow us to identify the integration site for the ras^H and myc in these cells in order to determine if integration is random or nonrandom.

3. We intend to investigate the induction of other gene products by the ras^H gene, particularly the autocrine motility factor described by Liotta et al., Proc. Natl. Acad. Sci. (in press).

Methods Employed:

Recombinant DNA methods, including gel electrophoresis, Southern blotting, plasmid manipulations, screening of λ library, genomic and cDNA, and $Ca(PO_4)_2$ mediated transfer of DNA. Immunoprecipitation, immunofluorescence of proteins, lung colonization metastasis and tumorigenicity assays in nude mice.

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 metastataic 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.

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.

The karyotypes of the transformed rat embryo fibroblasts was investigated. They were diploid or near diploid with a minor change. The karyotypes of the metastases and the tumors were identical to the parent cells indicating that no karyotypic selection took place. Since chromosomal instability followed by selection has been suggested as a model for tumor progression, these experiments indicate that this model is applicable to this system.

Significance to Biomedical Research and the Program of the Institute:

This system will allow us to explore the genetic basis of metastasis. Since metastasis is a frequent cause of treatment failure for cancer patients, any understanding of this process which could lead to diagnostic methods to predict metastases or lead to new treatments to prevent metastasis could be of great importance.

Proposed Course of Research:

1. Continue to develop methods to reliably induce the metastatic phenotype in nonmetastatic cells such as C127 transformed by ras^H using gene transfer.

2. Attempt to clone the gene(s) responsible for the induction of metastasis in C127 transformed by ras^H.

3. Attempt to identify genes induced by the ras^H gene which lead to metastatic behavior.

4. Continue the karyotypic analysis of ras^H transformed rat embryo fibroblasts including in situ hybridization.

Publications:

Muschel, R.J., Williams, J.E., Lowy, D., and Liotta, L.A.: Harvey ras induction of metastatic potential depends upon oncogene activation and the type of recipient cell. Am. J. Pathol. 121: 1-8, 1985.

Pozzatti, R.P., Muschel, R.J., Williams, J.R., Howard, B., Liotta, L.A., and Khoury, G.: Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. Science 232: 223-227, 1986.

Muschel, R.J., Nakahara, K., Chu, E., Pozzatti, R., and Liotta, L.A.: Induction of the metastatic phenotype does not require karyotypic change. Cancer Res. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08266-06 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Structure and Function of Basement Membrane Molecules

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

PI:	C.N. Rao	Visiting Associate	LP NCI
	U. Wewer	Visiting Fellow	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI
	I.M.K. Margulies	Biologist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The nature and assembly of basement membrane constituents namely IV collagen, laminin and heparan sulphate proteoglycan were studied using a variety of in vitro binding assays. These basement membrane macromolecules were isolated from the EHS tumor grown in C57 black mice. Protease-derived fragments of laminin and IV collagen were characterized by rotary shadowing electron microscopy. The domains required for binding of laminin and IV collagen were identified. Laminin is a cross-shaped molecule with three equal short arms and one long arm. The cell binding region of laminin was also identified and found to reside at the intersection of the three short arms. The carbohydrate composition of laminin was obtained and the distribution of sugars on the long and short arms of laminin molecule was studied. Type IV collagen is a rope-like structure (360 nm) with a globular domain at the carboxyterminal end and a disulphide-rich amino terminal end. A major binding site for laminin is identified at about 100 nm away from the globular end of type IV collagen. The binding domain on laminin for its receptor has now been isolated using protease treatment of laminin. A monoclonal antibody is shown to recognize this domain and block the binding of laminin to the receptor. A complete model has been developed for the orientation of the cell surface laminin receptor, laminin itself, and type IV collagen in the basement membrane.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

1. To prepare purified protease-derived fragments of basement membrane components. 2. To use these fragments to study the structure and function of basement membrane molecules. 3. To raise polyclonal and monoclonal antibodies to different domains of laminin molecule. 4. To quantitate laminin and fragments of laminin in normal and cancer tissues. 5. To isolate the smallest possible fragment of laminin that has a receptor binding domain.

Methods Employed:

1. Characterization of laminin

Laminin was purified from 0.5 M NaCl extracts of mouse EHS tumor by DEAE cellulose and stored frozen in phosphate-buffered saline, 4.0 M NaCl precipitation.

Protease-derived fragments of laminin were purified and examined by electron microscopy. The alpha fragment generated by digestion with α-thrombin lacks the long arm but retains the three short arms with globular end regions. The C_1 and P_1 fragments generated by chymotrypsin and pepsin, respectively, lack both the long arm and the globular end regions of the short arms. The C_1 fragment has "T" structure with arm lengths of 32 nm. The P_1 fragment is similar in appearance but has shorter arm lengths (26 nm). ^{14}C-IV collagen purified from EHS tissue was digested with pepsin (1:10 w/w) at 4°C for 16 hr. Pepsin destroys the 'NC1' domain of IV collagen leaving a 250 nm major triple helix and the '7S' domain intact.

2. Iodination of laminin and laminin fragments

Laminin and the three fragments were iodinated by the lacto-peroxidase method. The reagents and the unreacted iodine were removed from the iodinated proteins by molecular sieve chromatography.

3. Binding assays

A. To analyze the carbohydrate composition and distribution on laminin - Nitrocellulose filters (13 mm dia. 8 micron pore size Millipore SCWP type) were saturated with 20 µl of the lectin in phosphate-buffered saline (+ calcium + magnesium) pH 7.4 (PBS). Following lectin binding to the nitrocellulose, the filters were immersed in a blocking solution of 3% bovine serum albumin (BSA/PBS: 2 x 90 min) and rinsed with PBS (2 x 30 min). The blocked filters were saturated with ^{125}I-labeled whole laminin or the laminin fragments and incubation was conducted in a humidified chamber at 25°C for 30 min. The filters were washed with PBS (2 x 45 min) and placed in a Packard Gamma Counter to measure the bound radioactivity. The radioactivity bound to NC filters blocked with 3% BSA alone was subtracted as background (8-15%) to give the final value for bound radioactivity. Carbohydrate specificity of the lectin-laminin binding was studied by two means: In the first method, the laminin-lectin binding reaction was conducted in the presence of excess haptenic sugars and in the second

method, the haptenic sugars were added at the end of the binding reaction and the radioactivity was eluted from the filters over 18 hr at 25°C.

B. To study the binding of laminin and IV collagen - ^{14}C-pro IV collagen was soaked overnight in laminin sepharose and the unbound IV collagen was thoroughly washed. The bound IV collagen was successively eluted with 1.0 M NaCl and 6.0 M urea solutions. Pepsin digested IV collagen was also used to quantitate its binding to laminin sepharose column. Iodinated laminin and laminin fragments were employed to bind to IV collagen immobilized on SCWP Millipore filters. Alternatively, labelled IV collagen and its pepsin digest were used to quantitate their binding to laminin and laminin fragments coated onto SCWP Millipore filters.

Major Findings:

1. Two monoclonal antibodies to laminin (LAM-I and LAM-II) were used to localize the protease resistant T shaped C_1 or P_1 fragment of laminin. Both LAM-I and LAM-II react with the 200 KD subunit and C_1 fragment suggesting that C_1 fragment originates from the 200 KD subunit. Under mild reducing conditions, about 20% of the 200 KD subunit from laminin and 200 KD subunit retained in α-thrombin digests were identical and different from that of the 400 KD subunit. These data suggest that α-thrombin digestion of laminin can be used to isolate the 200 KD subunit in native form and that the protease resistant T-shaped intersection of the laminin short arms is embodies in the 200 KD subunit. The 200 KD subunit was subjected to further proteolysis resulting in a purified 50 KD fragment which competitively blocked the binding of laminin to the receptor. The monoclonal antibody LAM II reacts with this fragment and abolishes the binding of laminin to the receptor.

2. IV collagen binds laminin with a high affinity and 6.0 M urea was needed to elute the IV collagen bound to laminin. Binding assays demonstrated that the end globular domains on laminin short arms binds the pepsin sensitive carboxy terminal 'NCI' domain of IV collagen. The long arm of the laminin molecule binds the heparan sulphate proteoglycan.

Significance to Biomedical Research and the Program of the Institute:

Invasive neoplasia is accompanied by profound changes in the structure and amount of basement membranes. Study of the structure of basement membrane components and their interaction with cells may lead to therapeutic approaches which involve modification of tumor cell interaction with basement membranes.

Proposed Course of Research:

1) To sequence the binding domain on laminin for its receptor; 2) to characterize the binding domains on the NC1 domain type IV collagen molecule; 3) to study the assembly of basement membranes in vitro; and 4) to visualize (by EM) the binding of laminin, type IV collagen and proteoglycan together.

Publications:

Roberts, D.D., Rao, C.N., Magnani, J.L., Spitalnik, S.L., Liotta, L.A., and Ginsburg, V.: Laminin binds specifically to sulfated glycolipids. Proc. Natl. Acad. Sci. USA 82: 1306-1310, 1985.

Rao, C.N., Margulies, I.M.K., and Liotta, L.A.: Binding domain for laminin on type IV collagen. Biochem. Biophys. Res. Commun. 128: 45-52, 1985.

Liotta, L., Rao, C.N., and Wewer, U.: Biochemical interactions of tumor cells with the basement membrane. Ann. Rev. Biochem. 85 (in press)

Roberts, D.D., Rao, C.N., Liotta, L.A., Gralnick, H.R., and Ginsburg, V.: Comparison of the specificities of laminin, thrombospondin, and Von Willebrand factor for binding to sulfated glycolipids. J. Biol. Chem. (in press)

Wewer, U.M., Damjanov, A., Weiss, J., Liotta, L.A., and Damjanov, I.: Mouse endometrial stromal cells produce basement membrane components. Differentiation (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09127-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Effect of TPA on Type IV Collagenolytic Activity in Normal and Neoplastic Cells

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

PI:	M. Ballin	Visiting Fellow	LP NCI
OTHER:	U.P. Thorgeirsson	Visiting Scientist	LP NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

1. The variations in production of type IV collagenase were studied after adding the tumor promoter, TPA, to normal and metastatic cells. This study shows an increase in the secretion of type IV collagenase by human melanoma (A2058), fibrosarcoma (HT1080) and mouse skin fibroblast (NIH-3T3) cell lines. On the other hand, no increase in the production of type IV collagenase could be found after treatment with TPA of two different primary human fibroblast cell lines. The optimal concentration of TPA was also studied and the maximum stimulation was obtained with 10^{-8} to 5 x 10^{-7} M TPA.

2. A new type IV collagenolytic assay was developed using activated p-nitrophenyl glass beads. The glass beads were coupled with radiolabeled type IV collagen, stored at 4°C and then used for the assay. The binding of type IV collagen to the beads was studied with respect to tissue and temperature.

GPO 914-918

Project Description

Objectives:

1. To study the induction of type IV collagenase secretion by TPA in normal versus metastatic cells.

2. To develop a new and more sensitive assay to measure the type IV collagenolytic activity using labeled type IV collagen immunobilized on p-nitrophenyl activated glass beads.

Methods Employed:

1. Coupling of ^{3}H proline-labeled type IV collagen to p-nitrophenyl activated beads.

2. Type IV collagenase assay using labeled type IV collagen in solution or coupled with activated glass beads.

3. Extraction and labeling of type IV collagen from EHS tumor tissue.

4. Cell cultures of metastatic and normal cell lines and collection of media from them.

5. Treatment of semiconfluent 150 cm^2 cell culture flasks with different concentration of TPA.

Major Findings:

1. Addition of 10^{-9} to 10^{-6} M TPA to the serum-free medium of subconfluent human melanoma cells (A2058) stimulated the secretion of type IV collagenase.

2. The maximum stimulation of 500% on the secretion of this enzyme by the A2058 was obtained with 10^{-8}M TPA.

3. Increased activity was also obtained by the treatment with TPA of human fibrosarcoma cells (HT1080). The optimal concentration in this case was 5×10^{-7} M.

4. The same effect was obtained by using a normal, but easily transformed mouse skin fibroblast cell line (NIH-3T3).

5. Two different human fibroblast cell lines, one from fetal lung (IMPR.90), the other one from adult lung (MRC5) were examined, but variations were not detectable after adding of TPA.

6. Type IV collagenase assay was improved by the coupling of labeled type IV collagen to activated p-nitrophenyl glass beads. The sensitivity was increased 400% by using the immobilized ^{3}H-type IV collagen as compared to the soluble collagen used in standard type IV collagenase assay.

Significance to Biomedical Research and the Program of the Institute:

Type IV collagenase, produced by malignant cells, specifically degrades basement membrane collagen (type IV collagen) which is a major structural barrier of blood vessels and lymphatics for invasive tumor cells. This enzyme may be one of the most significant proteolytic enzymes, used by tumor cells during the metastatic process. In order to understand the mechanisms controlling regulation of type IV collagenase, we have used the tumor promoter, TPA, to stimulate its production in normal and malignant cells. So far the data indicate that TPA does not induce type IV collagenase production in primary cultures of normal adult fibroblasts or fetal fibroblasts. However, in NIH-3T3 fibroblasts which have been immortalized and do easily undergo spontaneous transformation, TPA stimulates the collagenase IV activity. This initial observation of the project suggests that type IV collagenase can only be induced by TPA in fibroblastic cells that have been immortalized or initiated, or in neoplastic cells.

Proposed Course of Research:

1. Study the effect of TPA on type IV collagenolytic activity of different types of normal fibroblasts.

2. Check if different degress of confluency of the primary fibriblast cultures affect the TPA induction.

3. Prepare mRNA from different cultures of TPA induced cells, normal and malignant. Check if TPA stimulates transcription of type IV collagenase mRNA, using a cDNA clone of the enzyme.

4. Quantitate TPA induction of type IV collagenase at different stages of carcinogenesis, using either epidermal cells or rat hepatocytes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09130-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Laminin Receptor in Breast Tissue, Benign and Malignant Tumors

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

PI:	G.J. Bryant	Expert	LP NCI
OTHER:	C.N. Rao	Visiting Associate	LP NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

R.R. Brentani, Ludwig Institute for Cancer Research, Sao Paulo, Brazil

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.8	0.2

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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

Project Description

Objectives:

1. Establish a standard protocol for collection of both human breast tissue and pertinent clinical data.

2. Determine specific laminin binding capacity of normal, benign and malignant human breast tissues.

3. Correlate the above results with clinical data.

Methods Employed:

In order to determine the binding capacity of available laminin receptor(s) in small breast carcinoma samples (i.e. less than 0.5 grams of tissue), we have successfully devised and employed an indirect radioimmune assay. The method uses as the primary antibody a monoclonal antibody raised against the laminin receptor from human breast carcinoma tissue (mouse IgM anti-human laminin receptor, MAbLR). Following appropriate incubation and washes, the secondary antibody, a radiolabeled rabbit α mouse IgM, is applied. A two hour incubation with 3% BSA is used to inhibit nonspecific binding.

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. Statistical analysis of these parameters is under way.

Significance to Biomedical Research and the Program of the Institute:

Laminin receptor binding may facilitate metastasis of tumor cells. The preliminary findings are supportive of the hypothesis that there are a large number of available receptors (i.e. without ligand) on invading tumor cells which may facilitate interaction of these cells with host membrane(s) during metastasis. If, in fact, this is the case, then one could envision an area of possible therapeutic intervention which would attempt to manipulate the availability of the receptors.

Proposed Course of Research:

Use the established indirect radioimmune assay of breast carcinoma plasma membranes and correlate the results with clinical information.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09131-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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:	U. Wewer	Visiting Fellow	LP NCI
	A.P. Claysmith	Biologist	LP NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

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Dr. M. Jaye, Meloy Lab., Springfield, VA

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Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.3	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 interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. To better understand the protein components that make up the extracellular matrix and how they are regulated, we have undertaken to construct, isolate, and characterize molecular clones of laminin receptor and of several different collagens. Laminin receptor is a cell surface protein to which laminin (a major component of basement membrane) specifically binds. Using a monoclonal antibody directed against the laminin-binding domain of laminin receptor, we screened a human endothelial cell cDNA λgt11 library. Six plaques showed an intense reaction with the anti-laminin receptor monoclonal but showed no reactivity toward a variety of control antibodies. The sizes of the cDNA inserts of the six clones ranged from 450 to 950 base pairs. Restriction enzyme mapping and Southern hybridization identified a 400 base pair fragment which was identical in each cDNA clone, suggesting that this fragment may represent the laminin-binding domain of laminin receptor. Definitive identification of the cDNA clones was based on comparison of cDNA sequence with the amino acid sequence of a cyanogen bromide-generated octapeptide of purified placental laminin receptor. The laminin receptor cDNA clone recognizes a 1700 base mRNA, which would be sufficient in length to code for a protein with the expected size of laminin receptor. The level of laminin receptor mRNA in a variety of human carcinoma-derived cell lines correlated with the number of laminin receptors on the cell surfaces of those cells. This suggests that the amount of laminin receptor mRNA may be a rate-limiting control step in the biosynthesis of laminin receptor, and hence in the regulation of cellular attachment to basement membranes via laminin.

Project Description

Objectives:

The objective of this project is to molecularly clone specific components of the extracellular matrix with which cells interact. Particular emphasis is placed on proteins that comprise or interact with the basement membrane. The integrity or lack thereof of the basement membrane surrounding tumors may be an important indicator of its essential invasiveness. Our immediate goals are to:

1. Isolate and identify a cDNA clone encoding laminin receptor, a protein on the cell surface to which laminin specifically binds.

2. Determine the protein sequences of laminin receptor by sequencing the cDNA clone. This will increase the understanding of the structure of the laminin receptor.

3. Study the regulation of laminin receptor synthesis and expression.

4. Isolate genomic clones and the promoter of the laminin receptor gene. Transfect the entire gene into cells to determine the effect of laminin receptor on cell behavior, specifically the ability of a cell to attach to basement membrane or to modulate its invasiveness and metastatic properties.

Methods Employed:

Laminin receptor was previously purified from the plasma membranes of metastatic breast carcinoma tissue and monoclonal antibodies were obtained and characterized by members of the Tumor Invasion and Metastases Section in collaboration with others. One such antibody, 2H5, specifically recognized laminin receptor on Western blots and could block binding of laminin to either plasma membranes or cells, or could block attachment of cells to amnion basement membrane. It presumably recognizes the laminin-binding domain of laminin receptor. Cyanogen bromide peptides of purified laminin receptor were purified and microsequenced to obtain authentic protein sequence.

A cDNA library was constructed at Meloy Laboratories using RNA template from human endothelial cells. The cDNAs were inserted into the EcoR1 site of phage λgt11 in such a way that the cDNA was fused to the end of the β-galactosidase gene. In this expression library, depending on orientation and reading frame, one in six phages could synthesize a fusion protein consisting of β-galactosidase and the protein fragment encoded by the cDNA. 1.5 million plaques of the recombinant λgt11 library were screened using the monoclonal antibody 2H5. Positive plaques were purified and tested for nonreactivity toward control antibodies. They were analyzed by restriction endonuclease mapping and Southern hybridization. The largest cDNA insert was subcloned into both pBR322 and pUC plasmids to facilitate cDNA sequencing. Both the Maxam and Gilbert chemical modification and the Sanger dideoxy synthesis sequencing methods were used.

To obtain genomic clones of laminin receptor as well as cDNA sequence more 5' to the first laminin receptor cDNA isolates, human genomic DNA libraries and cDNA libraries were screened using a restriction fragment of laminin receptor cDNA as a hybridization probe. Positive plaques were purified and analyzed by restriction endonuclease mapping and Southern hybridization.

The level of mRNA recognized by the cDNA insert was determined in a variety of tissues or cells by Northern hybridization.

Major Findings:

Characterization of a laminin receptor cDNA clone. Six plaques were selected after screening of the human endothelial cell cDNA λgtll library with the monoclonal antibody directed against the laminin binding domain of the laminin receptor. After plaque purification, all six clones showed an intense reaction with the anti-laminin receptor monoclonal antibody. The clones showed no reactivity toward a class-matched monoclonal antibody directed against human α-amylase, nor toward antibodies directed against proteins similar in size to laminin receptor, e.g. albumin. The sizes of the cDNA inserts of the six clones ranged from 450 to 950 base pairs. Restriction enzyme mapping and Southern hybridization identified a 400 base pair fragment which was identical in each cDNA clone. The coding sequence of this fragment may represent the antigenic domain recognized by the anti-laminin receptor monoclonal antibody. The cDNA insert of the largest clone, λELR4, was subcloned into pBR322 and pUC8 to facilitate DNA sequencing by the Maxam and Gilbert chemical modification method. In addition, the EcoRI-SacI fragment was subcloned into M13 for dideoxy sequencing. The entire cDNA sequence of λELR4 has been determined and reveals a 253 amino acid open reading frame consistent with the right arm EcoRI insertion site of the λgtll vector. The sequence contains a unique octapeptide which completely matches the amino acid sequence of a cyanogen bromide peptide generated from purified placental laminin receptor. Also within the cDNA sequence is a 6 amino acid repeat, separated by 3 amino acids, which is located toward the carboxy terminus of the laminin receptor. The cDNA sequences which encode the amino acid repeats are not, however, identical. Following the stop codon (UAA), the 3' untranslated region extends for 66 base pairs and includes the canonical polyadenylation signal AAUAAA 17 bases upstream from a long poly (A) stretch (approximately 150 bases).

Regulation of laminin receptor gene expression. Northern blot hybridization experiments have identified the size and relative abundance of the laminin receptor mRNA. The cDNA probe recognizes a single 1700 base mRNA which is sufficient in length to code for a protein with the estimated size (68 to 72 kilodaltons) of laminin receptor. The level of hybridized RNA from a variety of human epithelial cell lines correlates with laminin receptor assays (ability to bind to laminin). In particular, there was significantly greater hybridization to RNA from the metastatic breast carcinoma cell line MCF7 than to RNA from the nonmetastatic breast carcinoma cell line ZR75. This suggests that the synthesis of laminin receptor may be a rate limiting control step in the regulation of laminin attachment and in the metastatic process.

Isolation and characterization of full-length laminin receptor cDNA(s). The coding capacity of cDNA clone λELR4 (approximately 825 bp not including the poly (A) tail), is nearly 50% of the predicted size (1700 bases) of the laminin receptor mRNA. To obtain sequences upstream, cDNA libraries constructed from human endothelial and teratocarcinoma cells were screened with a 5' restriction fragment of λELR4. One isolate, λELR14, contains sequences 150 base pairs upstream from λELR4. Sequencing and further screening is in progress.

Isolation of human laminin receptor genomic clones. On Southern blots of genomic DNA from a variety of human cell lines and tissues, multiple DNA bands hybridize to laminin receptor cDNA, suggesting that there is a laminin receptor gene family. Two human genomic DNA libraries (obtained from A. Banks and T. Maniatis) were screened with laminin receptor cDNA sequences to initiate studies on the laminin receptor gene. Multiple plaques from both libraries were isolated and phage is currently being purified for analysis.

Significance to Biomedical Research and the Program of the Institute:

The interaction of highly metastatic tumor cells with the basement membrane is fundamentally different from the interaction of benign cells. The identification of a specific cell surface receptor to which laminin binds is a significant breakthrough in understanding of these interactions. Progress has been limited due to the difficulty in isolating sufficient quantities of laminin receptor. The molecular cloning of laminin receptor will provide us with sequencing data that will circumvent this problem. The ability to probe cells for mRNA content and to eventually obtain regulatory elements of the laminin receptor gene will also provide us with tools to understand how the synthesis of laminin receptor is regulated.

Proposed Course of Research:

Primary emphasis is being placed on the isolation of cDNA sequences upstream from the current laminin receptor cDNAs. In addition, the structure of the laminin receptor gene will be elucidated. The suggestion that there is a laminin receptor gene family will be further investigated by determining possible tissue-specific differences in laminin receptor as well as by analysis of the multiple isolates from the genomic DNA libraries. Regulatory studies to determine if the synthesis of laminin receptor is down-regulated in more differentiated cells will be undertaken to better understand how modulation of laminin receptor gene expression affects metastatic cell behavior.

Publications:

Sangiorgi, F.O., Benson-Chanda, V., de Wet, W., Sobel, M.E., and Ramirez, F.: Analysis of cDNA and genomic clones coding for the pro α1 chain of calf type II collagen. Nucl. Acids Res. 13: 2815-2826, 1985.

Huerre-Jeanpierre, C., Mattei, M.-G., Weil, D., Grzeschik, K.H., Chu, M.-L., Sangiorgi, F.O., Sobel, M.E., Ramirez, F., and Junien, C.: Further evidence for the dispersion of the human fibrillar collagen genes. Am. J. Hum. Genet. 38: 26-37, 1986.

Wewer, U.M., Liotta, L.A., Jaye, M., Ricca, G.A., Drohan, W.N., Claysmith, A.P., Rao, C.N., Wirth, P., Coligan, J.E., Albrechtsen, R., Mudryj, M., and Sobel, M.E.: Altered levels of laminin receptor mRNA in human carcinoma cells with varying ability to bind laminin. Proc. Natl. Acad. Sci. USA (in press)

Ramirez, F., Bernard, M., Chu, M.-L., Dickson, L., Sangiorgi, F., Weil, D., deWet, W., Junien, C., and Sobel, M.E.: Isolation and characterization of the human fibrillar collagen genes. Ann. N.Y. Acad. Sci. 460: 117-129, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09152-01 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Expression of H-ras gene in metastatic (NMU)-Induced Rat Mammary Carcinoma

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

PI:	U.P. Thorgeirsson	Visiting Scientist	LP NCI
OTHER:	M.E. Sobel	Senior Investigator	LP NCI
	M. Ballin	Visiting Fellow	LP NCI
	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

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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 1/2	1	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.)*

We have previously demonstrated that activated cellular Harvey (H) ras oncogene can confer the metastatic phenotype upon non-neoplastic NIH/3T3 cells through DNA transfection. The possibility was examined whether the metastatic behavior was associated with amplification or increased expression of the ras gene. An autochtonous NMU-induced rat mammary carcinoma that possesses activated Harvey ras was used for this purpose. Nonmetastatic vs metastatic primary tumors were examined, as well as primary tumors vs metastases. There were no differences between the ras specific DNA and RNA levels of the nonmetastatic and metastatic tumors. Ten individual lung metastases and a single primary tumor were resected from a Sprague-Dawley rat, and were expanded through subcutaneous transplantation in nude mice. There was a variability in the ras expression among the lung metastases. Some were several fold higher than the primary and three of the ten metastases had barely detectable RNA levels. Southern blot of EcoR1 restricted DNA from the primary rat tumor and pooled fragments of multiple lung metastases showed about ten-fold higher ras DNA levels in the primary tumor. However, after multiple passages of NMU tumors in syngeneic rats, no differences in the ras DNA levels were detectable. These data imply that H-ras gene amplification or increased expression is not necessary for established metastases. The primary S.D. rat NMU tumor exhibited additional H-ras sequences in PVU-II cleaved DNA that were not seen in normal rat mammary glands, or the nude mouse transplants of the same tumor.

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

Project Description

Objectives:

1. To evaluate whether the ras gene is associated with the metastatic phenotype through amplification or increased expression.

2. To look for polymorphism in the ras oncogene among the primary and metastatic NMU tumors.

3. To compare ras expression in NMU tumors and normal mammary glands.

Methods Employed:

1. Extraction of genomic DNA and total RNA from NMU-induced tumors, either autochthonous or transplanted.

2. Southern and Northern blot analysis of nucleic acids.

3. Preparation of a ras specific probe by isolation of a 2.8 kbp. Bam H-1 fragment from a rat ras gene.

4. Nick translation and ^{32}P-labeling of the ras probe.

Major Findings:

NMU-induced rat mammary carcinoma carries a mutation of the H-ras gene that involves glycine-glutamic acid substitution in the 12th codon. Although NMU-induced tumors are metastatic, we have obtained three primary tumors with metastases to different organ systems, and three transplantable tumors that are persistently metastatic in syngeneic rats. We have also collected numerous non-metastatic NMU tumors and mammary glands from normal, pregnant and lactating rats. So far, DNA has been isolated from all of the samples. There are no differences in the H-ras related DNA levels between the normal, pregnant and lactating mammary glands. Similarly, the H-ras DNA levels of the normal glands do not vary significantly from that of the neoplastic DNA, neither the non-metastatic nor the metastatic. When primary nude mouse transplants of ten lung metastases were analyzed and compared to the single primary tumor that they were derived from, a variability in both DNA and RNA levels was observed. Although the H-ras expression was several times higher in some of the metastases than the primary tumor, three of ten metastases expressed very low levels of the H-ras. A Southern blot of the primary rat tumor and pooled fragments of the lung metastases revealed ten-fold higher H-ras specific DNA levels in the primary tumor. However, DNA analysis of the NMU tumors that had been passaged numerous times in syngeneic rats showed minimal variability in the H-ras DNA levels both among and between primary tumors and metastases. Digestion of the tumor DNA with Pvu-II revealed additional H-related ras sequences in the primary NMU tumor that were lost after transplantation in syngeneic rats or nude mice, and were not seen in the normal mammary glands or in the nonmetastatic NMU tumors.

Significance to Biomedical Research and the Program of the Institute:

The role of activated oncogenes in tumorigenesis is still poorly understood. We have demonstrated in the NIH/3T3 system that the complete metastatic phenotype can be conferred upon the recipient cell line by transfection with activated ras oncogenes. The significance of this finding in the study on metastasis is two-fold. Firstly, it demonstrates that the capacity to metastasize can be introduced into non-neoplastic cells through genetic transfer, using either DNA from malignant cells, or isolated ras oncogenes. Secondly, ras gene mediated induction of the metastatic phenotype represents a powerful tool to study the mechanism by which cellular genes relevant to the metastatic process are turned on.

Proposed Course of Research:

1. Use synthetic H-ras oligonucleotide probe containing the glycine-glutamic acid encoding point mutation in the 12th codon. This probe will verify that all the NMU tumors and their metastases possess the activated H-ras, in contrast to the normal mammary glands.

2. Do in situ hybridization studies on histological sections of the NMU-induced tumors, using the H-ras probe. This technique will enable us to localize the rat's gene at a cellular level and quantitate its expression in tumors of different malignant behaviors.

3. To study further the amplified H-ras sequences in the primary metastatic NMU tumor.

Publications:

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., Talmadge, J.E., and Liotta, L.A.: Expression of oncogenes in cancer metastases. In Welch, D.R., Bhuyan, B.K., and Liotta, L.A. (Eds.): Cancer Metastasis: Experimental and Clinical Strategies. Progress in Clinical and Biological Research, Vol. 212. New York, Alan R. Liss, Inc., 1986, pp. 77-93.

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., Sobel, M.E., Talmadge, J.E., and Liotta, L.A.: Role of ras oncogenes in experimental models of metastasis. In Lapis, K., Liotta, L.A., and Rabson, A.S. (Eds.): Biochemistry and Molecular Genetics of Cancer Metastasis. Boston, Martinus Nijhoff Publ., 1986, pp. 55-63.

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., Ballin, M., and Liotta, L.A.: Methods to study ras oncogene-mediated induction of the metastatic phenotype. In Kaiser, H. (Ed.): Progressive Stages of Malignant and Neoplastic Growth. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00550-06 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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.A. Matis	Senior Investigator	MB NCI
	L.M. Neckers	Research Chemist	LP NCI

COOPERATING UNITS *(if any)*

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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.

This information is utilized to develop improved classifications of disease and to distinguish new clinicopathologic entities. It also will be used as a basis for potential immunotherapy or adjunctive immunotherapy in a program of autologous bone marrow transplantation.

Project Description

Objectives:

To determine the clinical importance of immunologic phenotype, and determine if it correlates with clinical presentation, stage, response to therapy or survival. To determine the correlation of immunologic phenotype with conventional morphology. To improve diagnosis and subclassification of human malignant lymphomas.

Methods Employed:

Cells derived from malignant lesions are analyzed with a large battery of monoclonal antibodies directed against lineage-restricted surface and cytoplasmic epitopes, surface receptors and growth factor receptors. Cells are analyzed using a fluorescence activated cell sorter which permits collection of quantitative data as to density of surface epitopes and permits distinction of heterogeneous cell populations in tumor cell suspensions. Cells are also analyzed using immunocytochemical methods in both frozen sections and cytocentrifuge smears. This permits morphologic correlations to be drawn and allows for topographical distribution of cells within tissues.

Cells are also studied for a variety of lysosomal enzymes which are particularly useful in identifying cells of mononuclear phagocytic origin.

Patients studied are clinically and pathologically staged and treated in a uniform manner on NCI protocols.

Currently being developed are techniques of in-situ hybridization to study oncogene expression of normal and neoplastic lymphoid cells, to be correlated with phenotypic characteristics.

Major Findings:

Immunophenotypic studies of six patients with angiocentric immunoproliferative disorders (lymphomatoid granulomatosis and angiocentric lymphoma) were studied. All cases demonstrated a mature T-cell phenotype with a predominance of helper cells. The patient with angiocentric lymphoma had an aberrant mature T-cell phenotype and Southern blot analysis of genomic DNA demonstrated a clonal rearrangement of the T-cell antigen receptor beta chain.

The clinicopathologic material from 23 patients with angiocentric immunoproliferative disorders was reviewed. The patients could be histologically classified into three groups: group 1 (9 patients) had little or no cytological atypia; group 2 (6 patients) had moderate cytologic atypia but retained a polymorphous cellular infiltrate; group 3 (8 patients) had monomorphic angiocentric lymphomas at presentation which were classified as diffuse, mixed, small and large cell type or diffuse large-cell immunoblastic.

Progression to malignant lymphoma following initial immunosuppressive therapy occurred in three of nine patients in group 1 and 4 of 6 patients in group 2. The supervening lymphomas were refractory to subsequent aggressive chemotherapy,

with only one patient achieving a complete remission. In contrast, 7 of 8 patients in group 3 achieved a complete remission with aggressive combination chemotherapy. These results indicate that patients with angiocentric immunoproliferative lesions are at substantial risk for progression to angiocentric lymphoma and that initial immunosuppressive therapy may compromise clinical responsiveness when progression to lymphoma occurs. Moreover, these data suggest that all of these lesions represent part of a spectrum and that the angiocentric immunoproliferative lesions and angiocentric lymphomas are a single clinicopathologic entity within the spectrum of post-thymic T-cell proliferations.

Co-existing Hodgkin's disease and mycosis fungoides have been rarely reported simultaneously or consecutively in the same patient. It has been controversial whether these truly represent two distinct histologic entities, or whether the occurrence of such putative cases of Hodgkin's disease represents mis-diagnosis. In a single case, we were able to phenotypically characterize both lymph nodes involved by Hodgkin's disease and skin involved by mycosis fungoides in an individual patient. The cutaneous infiltrate had a mature helper T-cell phenotype and lacked the Leu M1 and Hefi-1 antigens associated with Hodgkin's disease. In contrast, the Reed-Sternberg cells and mononuclear variants of the Hodgkin's lymph node manifested the phenotypic markers of Hodgkin's disease but lacked mature T-cell markers characteristic of mycosis fungoides. This case helps to document the co-existence of these two diseases in a single patient.

Two patients initially presenting with hypereosinophilia and systemic vasculitis were studied. Both patients ultimately developed peripheral T-cell lymphomas. The clinical, pathologic, and immunologic features of these cases were described.

Significance to Biomedical Research and the Program of the Institute:

This information helps to define the clinicopathologic spectrum of non-Hodgkin's lymphoma and leads to improved pathologic classification schemes. As phenotypic studies are correlated with conventional histologic ones, it is determined which histologic observations are accurate and in which cases phenotypic studies must be employed. Neoplastic expansions of tumor cells often permit the identification of normal cellular phenotypes not previously recognized, and lead to increased understanding of the normal immune system as well.

Proposed Course of Research:

New lymphoma patients accrued to the NCI will be prospectively studied. Patients with recurrent disease will be studied to address questions of constancy of phenotype, clonal evolution, and multiple neoplastic clones. Techniques for in-situ hybridization are being developed so that phenotypic parameters may be correlated with oncogene expression. Molecular probes will also be used for molecular characterization of immunologic phenotype. Phenotypic information will be utilized in a collaborative program with Dr. R. Steis and other co-investigators as a basis for adjunctive immunotherapy for autologous bone marrow transplantation.

Publications:

Braziel, R.M., Hsu, S.-M., and Jaffe, E.S.: Lymph nodes, spleen, and thymus. In Spicer, S.S., Garvin, A.J., and Hennigar, G.R. (Eds.): Application of Histochemistry to Pathologic Diagnosis. New York, Marcel Dekker, Inc., 1986, pp. 203-256.

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Foon, K.A., Maluish, A.E., Abrams, P.G., Wrightington, S., Stevenson, H.C., Alarif, A., Fer, M.F., Overton, W.R., Poole, M., Schnipper, E.F., Jaffe, E.S., and Herberman, R.B.: Recombinant leukocyte A interferon therapy for advanced hairy cell leukemia: therapeutic and immunologic results. Am. J. Med. 80: 351-356, 1986.

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Tsujimoto, Y., Jaffe, E., Cossman, J., Gorham, J., Nowell, P.C., and Croce, C.M.: Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. Nature 315: 340-343, 1985.

Tsujimoto, Y., Cossman, J., Jaffe, E.S., and Croce, C.M.: Involvement of the bcl-2 gene in human follicular lymphoma. Science 228: 1440-1443, 1985.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E.S., and Croce, C.M.: The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 229: 1390-1393, 1985.

Hsu, S.-M. and Jaffe, E.S.: Phenotypic expression of T lymphocytes in thymus and peripheral lymphoid tissues. Am. J. Pathol. 121: 69-78, 1985.

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O'Shea, J.J., Jaffe, E.S., Lane, H.C., MacDermott, R.P., and Fauci, A.S.: Peripheral T cell lymphoma presenting as hypereosinophilia with vasculitis: Clinical, pathologic, and immunologic features. Am. J. Med. (in press)

Simrell, C.R., Boccia, R.V., Longo, D.L., and Jaffe, E.S.: Coexisting Hodgkin's disease and mycosis fungoides: Immunohistochemical proof of its existence. Arch. Pathol. Lab. Med. (in press)

Longo, D.L., Young, R.C., Wesley, M., Hubbard, S.M., Duffey, P.L., Jaffe, E.S., and DeVita, V.T., Jr.: Twenty years of MOPP therapy for Hodgkin's disease. J. Clin. Oncol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00552-06 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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	Staff Fellow	LP NCI
	E. Lipford	Expert (terminates 6-30-86)	LP NCI
	J. Sundeen	Biotechnology Fellow (begins 7-1-86)	LP NCI
	S. Pittaluga	Visiting Fellow (terminates 6-30-86)	LP NCI
	P. Cohen	Biotechnology Fellow (begins 7-1-86)	LP NCI
	M. Uppenkamp	Guest Worker	LP NCI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2 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 standerd unreduced type. Do not exceed the space provided.)*

We have undertaken a series of investigations aimed at improving the precision of diagnosis and classification of human lymphoproliferative disease. Our approach is based on determination of lineage and differentiation through the use of monoclonal antibodies, flow cytometry and molecular genetics. As a result of these studies, we have outlined, in detail, the molecular genetic basis of the diagnosis of lymphoproliferative disease.

We have proven that monoclonality is not pathognomonic of malignancy since some nonmalignant lymphoid proliferations are clonal. In further investigations of the application of molecular genetics to the diagnosis of clonality in lymphoproliferations, we have discovered a critical limitation of the human T-cell gamma gene and have characterized the restricted repertoire of the gamma gene on polyclonal T cells.

Our investigations of precursor T-cell (lymphoblastic) neoplasms have revealed a recapitulation of normal early T-cell ontogeny. Clonal precursor T-cell neoplasms exhibit developmental orchestration of the rearrangement and expression of genes encoding antigen recognition in human T cells. We have developed mutants of pre-T cell lines which do not transcribe the T-alpha gene and have lost expression of the T3 complex present in the parental cells.

Despite advances in the cellular origins of non-Hodgkin's lymphoproliferative disease, the lineage and clonal derivation of Hodgkin's disease remains largely unknown. We have prepared highly enriched Hodgkin's cell suspension from several patients and have discovered evidence of immunoglobulin gene rearrangements in the Reed-Sternberg fractions. This seminal finding provides a foundation for analysis of the molecular biology of Hodgkin's disease.

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

Project Description

Objectives:

To accurately identify and diagnose lymphoproliferative diseases and determine their cellular lineage and derivation.

Methods Employed:

Flow cytometry, DNA and RNA hybridization, tissue culture, density gradient centrifugation.

Major Findings:

1. Combined genetic and phenotypic analysis can resolve significant diagnostic dilemmas in hematopathology.

2. Nearly all human B- and T-lymphoid malignant neoplasms are monoclonal. However, neoplastic B-cell clones are genetically unstable and show frequent clonal evolution.

3. The T-cell, B-cell and follicular center cell origin of diffuse aggressive lymphomas, have been confirmed by detection of appropriately rearranged T-cell receptor genes, immunoglobulin genes or rearrangement of the bcl-2 gene associated with the t(14;18) chromosomal translocation.

4. The lymphoproliferation and supervening lymphomas of angioimmunoblastic lymphadenopathy (AILD) are clonal and may be a consequence of disordered immunoregulation.

5. Normal lymphoid tissues and benign lymphoid hyperplasias show no evidence of monoclonality. However, they consistently contain the same seven rearranged T-gamma genes each corresponding to distinct variable domains.

6. Precursor T-cell neoplasms occupy sequential differentiation compartments during early T-cell development as shown by the coordinated expression of surface membrane molecules, T-cell receptor gene rearrangement and transcription of genes encoding the T-cell receptor-T3 complex.

7. Reed-Sternberg cells and their variants can be isolated from tissues involved by Hodgkin's disease and have been shown to contain immunoglobulin gene rearrangements.

Significance to Biomedical Research and the Program of the Institute:

Human lymphoproliferative disease encompasses a broad range of clinical and biological entities. Development of a biological basis for their diagnosis and classification provides for appropriate selection of therapy. Moreover, human lymphoid neoplasms serve as models for the investigation of lymphocyte differentiation, cellular lineage and clonal expansion. Our findings have

helped establish biological techniques, i.e., monoclonal antibodies and molecular genetics, as tools for diagnostic hematopathology.

Proposed Course of Research:

1. Relate characteristic phenotypes of lymphoproliferative clinicopathological processes to gene rearrangement, chromosomal translocation and monoclonality.

2. Probe histiocytic proliferations for rearrangement and expression of immunoglobulin genes and proto-oncogenes known to be activated in monocytes.

3. Analyze nucleic acids from purified Reed-Sternberg cells for amplification and rearrangements of proto-oncogenes and for expression of proto-oncogenes and antigen receptor genes.

4. Determine the in vitro inducibility of differentiation in precursor T-cell neoplasms.

5. Focus on the role of cell surface CD2, CD5 and CD7 as regulators of the activation of the expression of genes of the T-cell receptor-T3 complex. We have developed mutant subclones of pre-T cell lines which selectively lack expression of the T-cell alpha gene. Activation of alpha expression will be tested by manipulation of CD2, CD5 and CD7 with appropriate antibodies. Furthermore, we have constructed a full-length alpha cDNA expression system and will transfect mutant cells lacking alpha transcripts to determine if T-cell receptor expression can be reconstituted.

6. Based on our discovery of the gamma variable gene repertoire in polyclonal T cells, we will investigate whether certain V-gamma genes are selected in the immune response. Two examples to be analyzed are circulating T8(CD8) and cells of AIDS patients and cells of the NK-LAK system (cells provided by M. Lotz, Surgery Branch, NCI).

Publications:

Hecht, T., Longo, D.L., Cossman, J., Bolen, J.B., Hsu, S.-M., Israel, M., and Fisher, R.I.: Production of monoclonal antibody that selectively binds Reed-Sternberg cells. J. Immunol. 134: 4231-4236, 1985.

Fisher, R.I., Cossman, J., Diehl, V., and Volkman, D.J.: Antigen presentation by Hodgkin's disease cells. J. Clin. Invest. (in press)

Pittaluga, S., Raffeld, M., Lipford, E.H., and Cossman, J.: 3A1 (CD7) expression precedes T/ gene rearrangements in precursor T (lymphoblastic) neoplasms. Blood (in press, July 1986)

Cossman, J.: Diffuse aggressive non-Hodgkin's lymphomas. In Jaffe, E.S. (Ed.): The Surgical Pathology of Lymph Nodes. New York, W.B. Saunders, 1985, pp. 203-217.

Jaffe, E.S., Cossman, J., Neckers, L.M., Braziel, R.M., and Simrell, C.R.: Immunologic phenotypes of non-Hodgkin's lymphomas: Correlation with morphology and function. In Cavalli, F., Bonadonna, G., and Rozencweig, M. (Eds.): Malignant Lymphomas and Hodgkin's Disease: Experimental and Therapeutic Advances. Boston, Martinus Nijhoff, 1985, pp. 25-36.

Cossman, J., Bakhshi, A., and Korsmeyer, S.: Gene rearrangements applied to diagnostic immunopathology. In Rose, N.R., Friedman, H., and Fahey, J.L. (Eds.): Manual of Clinical Laboratory Immunology, 3rd Edition. Washington, D.C., American Society for Microbiology, 1986, pp. 168-173.

Jaffe, E.S. and Cossman, J.: Immunodiagnosis of lymphoid and mononuclear phagocytic neoplasms. In Rose, N.R., Friedman, H., and Fahey, J.L. (Eds.): Manual of Clinical Laboratory Immunology, 3rd Edition. Washington, D.C., American Society for Microbiology, 1986, pp. 779-790.

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Hecht, T.T., Longo, D.L., Cossman, J., Bolen, J.B., Hsu, S.-M., Israel, M., and Fisher, R.I.: Production and characterization of a monoclonal antibody that binds Reed-Sternberg cells. J. Immunol. 134: 4231-4236, 1985.

Tsujimoto, Y., Jaffe, E.S., Cossman, J., Gorham, J., Nowell, P.C., and Croce, C.M.: Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. Nature 315: 340-343, 1985.

Tsujimoto, Y., Cossman, J., Jaffe, E.S., and Croce, C.M.: Involvement of the bcl-2 gene in human follicular lymphoma. Science 228: 1440-1443, 1985.

Raffeld, M., Neckers, L., Longo, D.L., and Cossman, J.: Spontaneous alteration of idiotype in a monoclonal B-cell lymphoma. Escape from detection by anti-idiotype. N. Engl. J. Med. 312: 1653-1658, 1985.

Fisher, R.I., Cossman, J., Diehl, V., and Volkman, D.J.: Antigen presentation by Hodgkin's disease cells. J. Immunol. 135: 3568, 1985.

Braziel, R.M., Sussman, E., Neckers, L.M., Jaffe, E.S., and Cossman, J.: Induction of immunoglobulin secretion in follicular non-Hodgkin's lymphomas: Role of immunoregulatory T cells. Blood 66: 128-134, 1985.

Lipford, E.H. and Cossman, J.: Biological diagnosis of B-cell neoplasia. Cancer Invest. 4: 69-80, 1986.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E.S., and Croce, C.M.: The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 229: 1390-1393, 1985.

Matsushita, S., Robert-Guroff, M., Trepel, J., Cossman, J., Mitsuya, H., and Broder, S.: Human monoclonal antibody against an envelope glycoprotein of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. USA 83: 2672-2676, 1986.

Cossman, J.: T-cell neoplasms and Hodgkin's disease. In Berard, C.W. and Dorfman, R.F. (Eds.): Malignant Lymphoma, Leukemia and the Immune System. IAP Monograph, Williams and Wilkins, 1986. (in press)

Pittaluga, S., Cossman, J., Trepel, J.B., and Neckers, L.M.: Inhibition of immunoglobulin secretion, but not immunoglobulin synthesis, by a monoclonal antibody. In Reinherz, E.L., Haynes, B.F., Nadler, L.M., and Bernstein, I. D.: Leukocyte Typing II: Volume 2 - Human B Lymphocytes. New York, Springer-Verlag, 1986, pp. 473-481.

Cossman, J.: Immunologic markers and histopathology of diffuse large cell lymphoma. In Skarin, V. (Ed.): Advances in Cancer Chemotherapy: Update on Treatment for Diffuse Large Cell Lymphoma. Park Row Publ., 1986. (in press)

Cossman, J. and Raffeld, M.: Anti-idiotype in the therapy of B cell malignancies. In Bona, C. (Ed.): Elicitation and Use of Anti-Idiotypic Antibodies and their Biological Applications. CRC Press, Boca Raton, Florida. (in press)

Cossman, J. and Pittaluga, S.P.: Immunophenotype in the characterization and diagnosis of acute leukemia: lymphoid markers. In Stass, S.A. (Ed.): Acute Leukemia: Characterization and Diagnosis. Marcel Dekker, Inc. (in press)

Nagai, H., Fisher, R.I., Cossman, J., and Oppenheim, J.J.: Decreased expression of monocyte DR antigens in patients with Hodgkin's disease. J. Immunol. (in press)

Pittaluga, S., Raffeld, M., Lipford, E.H., and Cossman, J.: 3A1(CD7) precedes T-β gene rearrangements in precursor T-cell (lymphoblastic) neoplasms. Blood (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00850-04 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Clonal Evolution of Lymphoid Neoplasms

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

PI:	J. Cossman	Senior Investigator	LP NCI
OTHER:	M. Raffeld	Medical Staff Fellow	LP NCI
	S. Pittaluga	Visiting Fellow (terminates 6-30-86)	LP NCI
	M. Uppenkamp	Guest Researcher	LP NCI
	E. Lipford	Expert (terminates 6-30-86)	LP NCI
	R. Coupland	Guest Researcher (begins 7-1-86)	LP NCI

COOPERATING UNITS *(if any)*

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 1/2	2 1/4	1/4

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 previously demonstrated that human B lymphoid leukemias and lymphomas are monoclonal neoplasms which, although arrested during development, retain a finite capacity to differentiate when appropriately activated. These investigations allowed us to uncover the natural clonal evolution of follicular lymphoma in vivo by the use of anti-idiotype antibody. We have further identified the frequency of occurrence and mechanism of tumor clonal evolution in human lymphoid neoplasms.

By thorough restriction mapping of rearranged genes in B-cell lymphomas, we have discovered frequent clonal evolution during the course of disease. Genetic changes were a consequence of secondary effects such as deletion, switching or somatic mutation. Specifically, we found no evidence of second primary neoplasms (biclonality). Despite frequent alterations of immunoglobulin gene loci, the bcl-2 gene, involved in direct fusion to the J_H gene in the t(14;18) translocation, remains conserved and showed no restriction fragment size changes.

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

Project Description

Objectives:

To investigate clonal evolution of lymphoid neoplasms with respect to 1) the characterization of the genetic events resulting in altered immunoglobulin genes, 2) the impact of new idiotypes on escape from host immune recognition and 3) the correlation of clonal evolution and tumor progression.

Methods Employed:

Genomic DNA (Southern) hybridization, RNA (Northern) blots, cell culture, cytotoxicity, ELISA, monoclonal antibody immunostaining, flow cytometry.

Major Findings:

1. High frequency of clonal evolution in B-cell lymphomas resulted from secondary genetic changes of immunoglobulin genes.

2. Conservation of the translocated chromosome 18 element (bcl-2) despite clonal evolution of follicular B-cell lymphomas.

3. Malignant lymphoma arising in the oligoclonal lymphoproliferative disorder, AILD, is monoclonal.

4. Subclones of precursor T-cell lines spontaneously down-regulate T-cell receptor alpha gene transcription and surface membrane T3-complex expression.

5. Clonal analysis of T-gamma human gene rearrangements in normal T cells indicates a) as few as seven V-gamma genes exist, b) rearrangements occur very early in pre-T cell development and c) the likelihood of rearrangement of a given V-gamma gene may be fixed.

6. We have established a long-term, IL-2 dependent, T-cell acute leukemia cell line (1795) from an individual with ataxia telangiectasia. This cell contains a unique chromosomal translocation t(14;18) (q11;q22) which may involve the T-alpha gene on chromosome 14 band q11. This leukemia likely arose by clonal selection in a setting of frequent chromosomal breakage and translocation at the sites of T-cell receptor genes in T cells.

Significance to Biomedical Research and the Program of the Institute:

Indolent B-cell lymphomas remain largely incurable even though a clinical complete remission can be achieved in most patients with chemotherapy. Furthermore, recurrence following apparent remission remains as a major obstacle to the ultimate cure of high-grade lymphoma. Our results demonstrate that recurrence is not due to emergence of a second tumor but rather regrowth of the initial lymphoma and, therefore, in such instances therapy was ineffective at eradicating the tumor in its entirety. Genetic changes in immunoglobulin genes during disease are a consequence of the normal instability found at these loci. Such changes may alter idiotypes and disrupt the host's capacity for immune

recognition. This may account for the well documented waxing and waning during the natural course of this disease. Alternative means of therapy are being sought for follicular lymphoma patients and future clinical interventions could exploit such an immune response.

Proposed Course of Research:

1. Evidence for V-region joinings, C_H isotype switching or somatic mutation as a basis for clonal evolution will be investigated. DNA from those lymphomas lacking t(14;18) will be surveyed for translocations of other chromosomal elements.

2. Evidence of clonal evolution will be analyzed in DNA obtained from peripheral blood and bone marrow lymphocytes in patients with low-grade lymphoma.

3. Altered expression of bcl-2 will be analyzed during the course of disease to determine associations with histological/clinical progression and clonal evolution.

4. T-cell neoplasms will be further analyzed for spontaneous, in vivo, clonal evolution by searching for alterations of T-cell receptor gene configuration and expression.

5. We will investigate selection of individual V-gamma genes during clinical immune responses.

6. Our T-cell leukemia cell line (1795) derived from a patient with ataxia telagiectasia will be analyzed by chromosomal in situ hybridization for translocation of the T-alpha gene on normal chromosome 14q11 and for genes near chromosome 18q21, e.g. bcl-2 and myelin basic protein. DNA will be subjected to restriction analysis for rearrangements of appropriate genes. Somatic cell hybrids will be prepared for further confirmation of genes involved in the translocation. Regulation of expression of the involved genes will be analyzed. If these initial studies are successful and appropriate restriction fragments identified, the involved loci will be cloned and sequenced to investigate the fine features of the recombination.

Publications:

Raffeld, M., Neckers, L., Longo, D.L., and Cossman, J.: Spontaneous alteration of idiotype in a monoclonal B-cell lymphoma. Escape from detection by anti-idiotype. N. Engl. J. Med. 312: 1653-1658, 1985.

Braziel, R.M., Sussman, E., Neckers, L.M., Jaffe, E.S., and Cossman, J.: Induction of immunoglobulin secretion in follicular non-Hodgkin's lymphomas: Role of immunoregulatory T cells. Blood 66: 128-134, 1985.

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Cossman, J. and Raffeld, M.: Anti-idiotype in the therapy of B-cell malignancies. In Bona, C. (Ed.): Elicitation and Use of Anti-Idiotypic Antibodies and their Biological Applications. CRC Press, Boca Raton, Florida. (in press)

Pittaluga, S., Raffeld, M., Lipford, E.H., and Cossman, J.: 3A1(CD7) precedes T-β gene rearrangements in precursor T-cell (lymphoblastic) neoplasms. Blood (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00855-04 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

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.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To determine if HTLV-I, a human T-lymphotropic retrovirus, is associated with a distinct clinicopathologic entity and to determine if pathologic criteria can be developed to recognize this entity.

Methods Employed:

Screening of sera for antibodies to the viral core proteins p19 and p24. Isolation of virus from tumor cells. Pathologic review of all positively identified samples.

Major Findings:

HTLV-I is associated with a distinct clinicopathologic syndrome. Although there is some overlap with other mature T-cell malignancies, some unique features have been identified. These include a leukemic pattern of lymph node infiltration and a broad spectrum of cytologic types. Although the classical syndrome of adult T-cell leukemia is a fulminant disease poorly responsive to chemotherapy, chronic forms of HTLV-associated disease have been more recently identified. So-called smoldering ATL is associated with chronic cutaneous manifestations, circulating atypical cells and an absence of hypercalcemia, hepatosplenomegaly and significant lymphadenopathy.

Significance to Biomedical Research and the Program of the Institute:

The recognition of defined clinicopathologic criteria for the identification of HTLV-I-associated malignancies should aid in diagnosis and further definition of the epidemiology of this disease.

Proposed Course of Research:

Analysis of prospective series of lymphomas in parts of the world endemic for HTLV-I.

Publications:

Jaffe, E.S., Clark, J., Steis, R., Blattner, W., Macher, A.M., Longo, D.L., and Reichert, C.M.: Lymph node pathology of HTLV and HTLV-associated neoplasms. Cancer Res. (Suppl.) 45: 4662s-4664s, 1985.

Jaffe, E.S., Katz, D., and Macher, A.M.: The pathology of the acquired immunodeficiency syndrome. In Broder, S. (Ed.): AIDS: Clinical and Research Perspectives. New York, Marcel Dekker, Inc. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00881-05 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Regulation of Cell Growth by Transferrin Receptors

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

PI:	L.M. Neckers	Expert	LP NCI
OTHER:	J.B. Trepel	Biologist	NMOB NCI
	S. Bauer	Graduate Student	LG NCI
	R. Nordan	Staff Fellow	LG NCI
	O. Colamonici	Visiting Fellow	LP 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:
3.5	3.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

All cells studied to date require transferrin for growth. We and others have shown that antibodies to the transferrin receptor block the growth of lymphoblastoid cell lines. In mitogen-stimulated lymphocytes, these antibodies block proliferation. We are studying the processes which regulate the appearance of these receptors in lymphocytes and lymphoblastoid cell lines, and the function of these receptors in cell growth and metabolism. We have demonstrated that G_1 arrest occurs in both normal and malignant cells when transferrin receptors are blocked, even if cells are expressing high levels of growth factor receptor messenger RNA and c-myc and c-myb messenger RNA. Furthermore, either blockade of calcium channels or addition of cAMP to cells results in G_1 arrest and loss of transferrin receptor mRNA. The effect of cAMP can be detected at the level of transcription.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Objectives:

To discern the regulation of transferrin receptor appearance in mitogen-stimulated peripheral blood lymphocytes as well as transformed cell lines, and to study the role of these receptors in proliferation and growth.

Methods Employed:

Quantitation of transferrin receptors is made by use of the cell sorter. DNA, RNA and protein synthesis are measured by incorporation by cell of radioactive substrates. Cellular DNA and RNA are studied using various molecular probes following standard techniques.

Major Findings:

1. Normal cells regulate transferrin receptor appearance by carefully controlled tissue-specific growth factors and their receptors. This regulation is lost in malignant cells and transferrin receptor expression becomes constitutive.

2. Nevertheless, both growth factor-dependent and constitutive transferrin expression is calcium dependent, while transcription of the IL-2 receptor gene and genes for c-myc and c-myb are not calcium dependent.

3. Transferrin receptor redistribution and its associated hyperphosphorylation can be dissociated by blockade of calcium channels. Hyperphosphorylation is not calcium dependent, but redistribution is.

4. Redistribution of transferrin receptors may reduce surface receptor levels by 60% without affecting proliferation.

5. Cyclic AMP terminates transferrin receptor gene transcription in normal and some malignant cells. This is correlated with a G_1 arrest.

6. Growth factor dependent mouse plasmacytoma cell transferrin receptor synthesis and mRNA expression depends on continuous presence of growth factor. Removal of growth factor results in loss of receptor mRNA and G_1 arrest.

Significance to Biomedical Research and the Program of the Institute:

What triggers a cell to divide is currently under intense investigation. Learning in a normal cell what this trigger(s) is(are), would be a monumental step in the understanding of cancer. Since the transferrin receptor is present on every type of dividing cell studied to date, and since its removal prevents cell division, we feel that studying the regulation of this receptor in normal and transformed lymphocytes will add to the understanding of what regulates cell division.

Proposed Course of Research:

We plan to continue our studies in two basic areas: 1) the role of the transferrin receptor in cell growth (using transformed cell lines) and 2) the role of the receptor in activation, proliferation and differentiation of normal lymphoid cells. We are currently investigating the relationship of transferrin receptor expression to various oncogenes and their products to determine if oncogene activation is needed for transferrin receptor expression in normal and malignant cells. This is in collaboration with Drs. M. Potter and F. Muchinsky of NCI. We are also focusing on regulation of transferrin receptor gene transcription using a full length cDNA probe. We will employ transfection studies as well as use of anti-sense constructs to study the requirements for the TfR in cell proliferation.

Publications:

Trepel, J.B., Klausner, R.D., Colamonici, O.R., Pittaluga, S., and Neckers, L.M.: Down-regulation of promyelocytic cell transferrin receptor expression by cholera toxin and cyclic adenosine monophosphate. In Reinherz, E.L., Haynes, B.F., Nadler, L.M., and Bernstein, I.D. (Eds.): Leukocyte Typing II: Volume 3. New York, Springer-Verlag, 1986, pp. 327-338.

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Neckers, L.M., McGlennen, R., and Colamonici, O.: Phorbol ester induced surface transferrin receptor modulation: No correlation with decreased cell proliferation. Exp. Cell Res. (in press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09146-01 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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 professionel personnel below the Principal Investigetor.) (Name, title, leboretory, and institute affiliation)*

PI:	L.M. Neckers	Expert	LP NCI
OTHER:	R. Heikkila	Visiting Fellow	LP NCI
	S. Bauer	Graduate Student	LG NCI
	R. Nordan	Staff Fellow	LG NCI
	J. Trepel	Biologist	NMOB 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 stenderd 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.

GPO 914-918

Project Description

Objectives:

To study the molecular regulation of the TfR at the transcriptional and post-transcriptional level by iron and other intracellular signals.

Methods Employed:

Common methods to study cellular RNA and DNA; FACS analysis to study receptor number and cell cycle distribution.

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.

Significance to Biomedical Research and the Program of the Institute:

Regulation of the TfR has been shown to be critical for normal cell growth. If one can ascertain the mechanisms at the molecular level which regulate the TfR gene, one will have a better understanding of normal growth regulation as well as what may lead to uncontrolled proliferation.

Proposed Course of Research:

1) We will study the ability of intracellular iron to regulate the truncated TfR mRNA in plasmacytoma cells. This will tell us if the 3' untranslated portion of the full-length mRNA is involved in the ability of iron to regulate TfR mRNA levels.

2) We will study the relationship between c-myc transcription and TfR transcription in HL-60 cells using the cAMP model. We will infect these cells with a c-myc cDNA construct under LTR control and study the ability of cAMP to differentiate these cells as well as shut-off the TfR gene. We will also infect with a similar construct containing TfR cDNA to study whether c-myc expression is still necessary for growth and whether cAMP can still cause cellular differentiation.

Publications:

Neckers, L.M., Nordan, R., Bauer, S., and Potter, M.: Studies on transferrin receptor expression in mouse plasmacytoma cells. Curr. Top. Microbiol. Immunol. (in press)

Trepel, J.B., Klausner, R.D., Colamonici, O.R., Pittaluga, S., and Neckers, L.M.: Down-regulation of promyelocytic cell transferrin receptor expression by cholera toxin and cyclic adenosine monophosphate. In Reinherz, E.L., Haynes, B.F., Nadler, L.M., and Bernstein, I.D. (Eds.): Leukocyte Typing II: Volume 3. New York, Springer-Verlag, 1986, pp. 327-338.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09147-01 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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	Expert	LP NCI
OTHER:	C.A. Vidal-Soto	Guest Researcher	LP NCI
	O.R., Colamonici	Visiting Fellow	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:
2	2	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have 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.

GPO 914-916

Project Description

Objectives:

To study the defective TfR in HTLV-I infected human T cells in order to obtain insight into the normal regulation of the TfR.

Methods Employed:

Standard techniques to study RNA and DNA including RNAse protection assays, primer extension methods and DNA sequencing and cloning. Receptor levels are determined by standard radiolabelled ligand methods as well as FACS analysis.

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.

Significance to Biomedical Research and the Program of the Institute:

While study of this problem may uncover some of the underlying mechanisms in HTLV-induced cellular transformation, these cells also serve as a natural model to study how TfR structure is involved in its own internalization. This in turn would help clarify the natural regulation of the TfR.

Proposed Course of Research:

Using primer extension methods, cDNA cloning and RNAse protection techniques, we will look for minor alterations in the TfR mRNA of HTLV-I infected cells. These may be single base changes removing a phosphorylatable amino acid from the cytoplasmic domain of the protein. Using site directed mutagenesis, we will try to insert a defective TfR protein in normal cells to study its behavior in comparison to the TfR of HTLV-I infected cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09148-01 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Mechanisms of Megakaryocytic Differentiation of K-562 Cells

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

PI:	L.M. Neckers	Expert	LP NCI
OTHER:	O.R. Colamonici	Visiting Fellow	LP NCI
	J.B. Trepel	Biologist	NMOB NCI

COOPERATING UNITS *(if any)*

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:
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.)*

We have shown that the synthesis of DNA is not only a phenomenon seen in S phase but also in G1 and G2 phases of the cell cycle in K-562 cells after TPA treatment. This DNA synthesis is probably due to megakaryocytic differentiation and consequent increase in the ploidy of these cells. We have demonstrated that once the cells are differentiated, they express large amounts of c-sis transcripts with concurrent shut-off of the c-myc gene. In the case of c-sis, the regulation of the gene is at the transcriptional level, while c-myc is under post-transcriptional regulation.

GPO 914-918

Project Description

Objectives:

To discern the role of c-myc in proliferation and its regulation during the differentiation process, and cDNA cloning of the A-chain of PDGF which is expressed in megakaryocytes (and TPA-differentiated K-562 cells).

Methods Employed:

Transfection of cells by retroviral vectors. cDNA cloning techniques and electrophoresis of DNA and RNA followed by hybridization with labeled probes.

Major Findings:

1) TPA treatment of K-562 cells results in G1 and G2 arrest of the cells with continued DNA synthesis.

2) TPA-treated K-562 cells resemble megakaryocytes by morphology and phenotypic analysis.

3) K-562 cells differentiated toward megakaryocytes express c-sis and c-fos.

4) c-myc is regulated at the transcriptional level during megakaryocytic differentiation.

5) By hybridizing with an oligonucleotide probe, we found that the A-chain of PDGF (PDGF-1) is expressed in these cells.

6) The infection of K-562 cells with a retroviral vector carrying c-myc blocks the effect of TPA at concentrations of 10^{-9}M but this agent is still effective at 10^{-8}M and 10^{-7}M, demonstrating a competitive interaction between c-myc expression and TPA-induced differentiation.

Significance to Biomedical Research and the Program of the Institute:

The fact that PDGF is found as a heterodimer in serum but PDGF is a B-chain homodimer in tumor cells, may explain the difference between the normal growth factor and its form found in tumor cells and could help to clarify its tumorigenic potential. Since the A and B chain are coded by genes on different chromosomes, defective coordinate regulation might result in expression of a homodimer instead of a heterodimer. TPA-treated K-562 cells serve as a useful model to study these processes.

Proposed Course of Research:

We plan to continue our studies in two areas: 1) cloning the A-chain of PDGF and studying its coordinate regulations with the B chain (c-sis) following TPA-induced differentiation of K-562 cells; and 2) elucidating the importance of c-myc and c-fos expression in relation to megakaryocytic differentiation of K-562 cells.

Publications:

Colamonici, O.R., Trepel, J.B., and Neckers, L.M.: Phorbol ester enhances deoxynucleoside incorporation while inhibiting proliferation of K-562 cells. Cytometry 6: 591-596, 1985.

Colamonici, O.R., Trepel, J.B., and Neckers, L.M.: Megakaryocyte differentiation: Studies at the molecular level using the K-562 cell line. In Levine, R.F., Williams, N., Levin, J., and Evatt, B.L. (Eds.): Megakaryocyte Development and Function. Progress in Clinical and Biological Research, Vol. 215. New York, Alan R. Liss, 1986, pp. 187-191.

Colamonici, O.R., Trepel, J.B., Vidal, C.A., and Neckers, L.M.: Phorbol ester induces c-sis gene transcription in stem cell line K-562. Mol. Cell. Biol. 6: 1847-1850, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09149-01 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

LAK Cell Leukemias

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

PI:	L.M. Neckers	Expert	LP NCI
OTHER:	O.R. Colamonici	Visiting Fellow	LP NCI
	J.B. Trepel	Biologist	NMOB NCI
	P.R. Brayton	Staff Fellow	IR,SN NINCDS
	L. Grimm	Senior Investigator	IR,SN NINCDS

COOPERATING UNITS *(if any)*

NMOB, NCI; IR,SN, NINCDS

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have studied 3 cases of leukemias with mixed phenotype and 2 lymphoblastic lymphomas trying to further characterize them. These cells respond to treatment with IL-2 as do normal LAK cells, in that they develop the ability to kill tumor cells. Their phenotype changes to resemble that of the mature LAK cell. Using these cases, we propose that the normal LAK progenitor cell is a T/myeloid stem cell which can be differentiated by IL-2 to become a mature LAK cell, while losing its myeloid characteristics.

GPO 914-918

Project Description

Objectives:

1) Characterize a potential new group of T-cell malignancies by their LAK cell-like killing activity following IL-2 exposure.

2) Discern the cytogenetic characteristics of these leukemias.

3) Study rearrangement and expression of different chains of the T-cell receptor (gamma, alpha, beta).

Methods Employed:

Fluorocytometric analysis. Southerns and Northerns to study the genomic organization of the T-cell receptor. LAK cell killing assays.

Major Findings:

1) We found T-cell malignancies capable of IL-2 induced LAK cell killing.

2) The phenotype analyzed by using monoclonal antibodies showed similarity among all five cases (T/myeloid stem cell).

3) Some cases with similar phenotype did not show this kind of killing activity.

4) Most of these cases don't rearrange the chain of the T cell receptor.

Significance to Biomedical Research and the Program of the Institute:

These studies help to further clarify mixed phenotype leukemias and provide potential new tools for their classification. The fact that IL-2 stimulates this cell opens a question of whether these patients might be treated with this lymphokine. Finally, the establishment of a LAK cell line will aid in the elucidation of the mechanism of LAK killing.

Proposed Course of Research:

1) Further characterization of these leukemias/lymphomas by cytogenetic studies.

2) Study the structure and expression of the different chains of the T-cell receptor.

3) Develop a cell line to facilitate the study of LAK cells.

4) Delineate the origin of the normal LAK cell.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09150-01 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Induction of Monocytic Differentiation by Sphingomyelinase

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

PI:	O.R. Colamonici	Visiting Fellow	LP NCI
OTHER:	L.M. Neckers	Expert	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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 standerd unreduced type. Do not exceed the spaca provided.)*

We have demonstrated that the enzyme sphingomyelinase is able to induce differentiation of the promyelocytic cell line HL-60. This new pathway for monocytic differentiation may explain the ability of TPA to differentiate HL-60 cells. Differentiation is mediated by the breakdown product of sphingomyelin:phosphorylcholine. Preliminary evidence suggests that the phorbol ester, TPA, differentiates HL-60 cells via stimulation of sphingomyelinase and not via C-kinase.

Project Description

Objectives:

To discern new mechanisms of tumor cell differentiation involving alteration of membrane lipid constituents.

Methods Employed:

Fluorocytometric analysis. Enzymatic reactions to measure sphingomyelinase activity and C kinase.

Major Findings:

1) HL-60 cells cultured in serum-free medium supplemented with ITS showed differentiation toward the monocytic lineage.

2) For further evaluation of the underlying mechanism, HL-60 cells were treated with phosphorylcholine, a breakdown product of sphingomyeline. The effect of phosphorycholine was similar to that of sphingomyelinase.

3) No effect was obtained with choline or ethanolamine.

4) The cells are not only differentiated but also functionally activated as is shown by an increase in phagocytosis.

Significance to Biomedical Research and the Program of the Institute:

The study of differentiation and its mechanisms can provide an important tool for understanding the blockage of tumor cells at a particular stage of differentiation. So far, all the mechanisms by which drugs like TPA work are poorly understood and this system could offer new insights toward understanding this problem.

Proposed Course of Research:

We plan to continue our studies concerning the mechanisms by which sphingomyelinase differentiates HL-60 cells, and we will try to link this system with the effects of TPA and DMSO which produce similar results. It will also be assessed which oncogenes are related with proliferation and differentiation of these cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09151-01 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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.

GPO 914-918

Project Description

Objectives:

To study the immunopathology of LAK-IL2 treated tumors and determine if immunophenotypic findings of either the tumor or infiltrating cells correlate with clinical response. To help determine the mechanism of action of LAK-IL2 treatment. To determine if these data can be used to predict in advance of treatment tumors that will be responsive to LAK-IL2.

Methods Employed:

Human tumor biopsy specimens are obtained before, during, and after conclusion of LAK-IL2 treatment. Biopsy specimens are snap frozen and then studied by immunohistochemical techniques. A battery of monoclonal antibodies are employed which can be used to phenotype not only the tumor cells but the infiltrating effector cells. Phenotypic markers for T cells, B cells, macrophages, and NK cells are used as well as markers which identify membrane receptors indicative of cellular activation. The use of an immunohistochemical technique permits not only quantitation of infiltrating effector cells but also enables one to observe the topographical distribution of cells within tissue; i.e., are effector cells located in the interstitium or are they intimately associated with tumor cells?

Immunophenotypic data are correlated with conventional clinical and pathologic parameters. Patients are treated in a uniform manner on approved NCI protocols.

Major Findings:

Patients showing an objective clinical response following LAK-IL2 treatment, exhibit a marked lymphoid infiltrate in tumor specimens. The infiltrate is composed predominantly of CD8 positive cytotoxic suppressor T lymphocytes. A significant infiltrate of NK cells or B cells is not observed. The infiltrate is frequently associated with tumor necrosis and the necrosis is associated with infiltration of macrophages as well. In responding tumors the infiltrating effector cells are intimately associated with the tumor cells and surround them on an individual cell basis. In nonresponding tumors a significant cellular infiltrate is not observed, and those cells that are present are present in the fibrovascular stroma surrounding the tumor and not in intimate association with tumor cells. The induction of HLADR on human malignant melanoma cells appears to correlate closely with the infiltration by effector cells. Tumors that do not show HLADR fail to contain a significant cellular infiltrate.

Significance to Biomedical Research and the Program of the Institute:

This information will effect the future development of clinical protocols and may be used to predict which tumors may respond to LAK-IL2. By identifying which cells are acting as effector cells, more specific modes of immunotherapy may be developed.

Proposed Course of Research:

The number of patients thus far studied is small. This study has only been initiated within the past six months. Additional patients need to be studied to determine if preliminary observations are maintained. Tumors studied to date include primarily malignant melanomas, which are known to develop HLADR following interferon in vitro. The study will be expanded to include other types of tumors, including malignant lymphoma and hypernephroma.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09144-02 LP

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Identification of Sequence Specific DNA Binding Proteins

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

PI: D.L. Levens 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:
2/3	2/3	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have been applying a technique developed by us (Levens, D. and Howley, P., Mol. Cell. Biol. 5: 2307-2316, 1985) for the rapid identification of sequence specific DNA binding proteins to examine mammalian transcriptional regulatory factors. By combining this technique with a sensitive exonuclease footprinting assay (Wu, C., Nature 317: 84-87, 1985), DNA-protein complexes can be formed, rapidly separated from the vast majority of protein and competitor nucleic acids, centrifugally concentrated and "footprinted".

This approach has led to the identification of the existence of a cellular factor which binds to the enhancer of the gibbon ape leukemia virus (GALV). The binding of this factor is eliminated in competitive binding assays only by nucleic acids which contain sequences highly homologous to the 48 bp repeated element present in the LTR of the Seato strain and which display enhancer activity in vivo.

Another application of this method has been in the study of the regulation of the human c-myc oncogene, in collaboration with the laboratory of J.M. Bishop at the University of California, San Francisco. Using a similar approach, we have demonstrated that there is a region upstream of the c-myc oncogene which negatively regulates transcription from promoter P1 and P2. The same region which negatively regulates, as measured in transfection assays, binds a factor which can be measured and enriched by the procedure outlined above.

GPO 914-916

Project Description

Objectives:

1. Identification of sequence specific trans-acting factors transcriptional regulatory molecules

   A. Gibbon ape leukemia virus enhancer binding protein
   B. Negative regulator of c-myc

2. Development of immunological and nucleic probes for studying the expression and regulation of these trans-acting factors.

3. Development of in vitro transcription systems responsive to trans-acting factors to help define the mechanism of their action.

4. Use of site specific mutagenesis and synthetic oligonucleotides to define the sequences necessary for in vivo and in vitro function of the trans-acting factors.

Methods Employed:

A variety of methods will be used. We will rely upon our method (cited above) and recent modifications of it as well as conventional chromatographic techniques and FPLC chromatography as necessary. In addition, a variety of recombinant DNA methods coupled with transfection assays will be used. Standard immunological approaches will be employed when we have purified enough antigen to raise polyclonal antibodies and/or monoclonal antibodies to the transcriptional regulatory molecules which we identify.

Significance to Biomedical Research and the Program of the Institute:

The regulation of gene expression is a central problem in molecular biology. Recombinant DNA technology and efficient transfection procedures have allowed the definition of DNA sequences which regulate vital physiological processes; this technique allows the identification of some mediators of these processes which could potentially greatly increase our understanding of gene regulation. Because aberrant gene expression is increasingly recognized as an important contributor to pathologic events, including neoplasia, a knowledge of mechanisms of transcriptional regulation is necessary for a complete understanding of many disease processes.

Proposed Course of Research:

See Methods Employed.

Publications:

Levens, D. and Howley, P. M.: Novel method identifying sequence-specific DNA-binding proteins. Mol. Cell. Biol. 5: 2307-2315, 1985.

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY REPORT
DCBD, NCI
October 1, 1985 through September 30, 1986

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:

During the past year 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 in secondary cultures. 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 alpha and beta chain message for the T cell antigen receptor. Furthermore we have cultured these cells from nude mice and have found that they express the entire T cell antigen receptor on their membrane. These cells may therefore represent an extra-thymic source of T cells in nude as well as normal mice.

We have also found that epidermal Langerhans cells (in contrast to keratinocytes) express little or no major histocompatibility Class I antigen. The deficient expression of Class I antigens permitted highly purified Langerhans cell populations to be isolated from epidermal cell suspensions by treatment with anti Class I antibody and complement. It is likely that the uniquely low cell surface expression of Class I antigen by Langerhans cells has relevance to both the immune response in the skin as well as to mechanisms of skin allograft rejection.

Molecular Basis of Autoimmune Skin Diseases:

This laboratory was recently established to study autoantibody-mediated skin diseases in order to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis and epidermal basement membrane zone (BMZ). Specifically, antibodies in these diseases define molecules in the normal epidermis. Using immunoprecipitation techniques and patient sera we have found that patients with epidermolysis

bullosa acquisita (EBA) have autoantibodies against a 290,000 dalton polypeptide, which is synthesized by both human keratinocytes and human dermal fibroblasts. A 145,000 dalton polypeptide that is detected by immunoblotting with EBA sera is not a precursor of the 290,000 dalton protein, and is probably a degradation product. In addition we have demonstrated that autoantibodies from both bullous pemphigoid and EBA patients that bind to the respective non-denatured antigens by immunoprecipitation may not bind to the antigens that have been denatured for immunoblotting. In other studies, we have been able to characterize the pemphigus foliaceus (PF) antigen by immunoblotting and have found that a monoclonal antibody against desmoglein I, a core desmosomal glycoprotein, binds to the antigen. This finding provides further proof for the hypothesis that a subgroup of PF patients have autoantibodies against a desmosomal glycoprotein. Sera obtained from patients with fogo selvagem, a form of pemphigus endemic to Brazil, have similar antigenic specificities to sera from patients with sporadic North American or European PF.

Detection and Analysis of Circulating Immune Complexes and Complement Proteins in Inflammatory and Immunological Processes:

We have continued our studies of the role of complement derived anaphylatoxins in the inflammatory response in general and immunologically mediated skin diseases in particular. Since cutaneous necrotizing vasculitis is a prototype of immunologically-mediated skin disease we have also studied the role of endothelial cells. We have succeeded in isolating and purifying to homogeneity the human anaphylatoxins C5a and C3 and have conducted extensive clinical testing of this material in the skin of humans. The inflammatory response to C5a is characterized clinically by marked wheal and flare reactions and histologically by mast cell degranulation and infiltration of blood vessel walls and surrounding skin by polymorphonuclear neutrophils. We have demonstrated the importance of cutaneous mast cells in the inflammatory process by showing abrogation of wheal and flare reactions to C5a in individuals who have had selective depletion of their cutaneous mast cells by local treatment with corticosteroids but interestingly still show marked infiltration of skin test sites with polymorphonuclear neutrophils. We have also shown the importance of the neutrophil in immediate hypersensitivity reactions by demonstrating a selective diminution of skin test reactivity to C5a in patients with bone marrow failure. We have also demonstrated that C5a will cause upregulation of immunoglobulin (Fc IgG) and complement receptor (CR1 and CR3) and monocytes and polymorphonuclear neutrophils indicating that C5a not only causes chemataxis of these cells at sites of complement mediated inflammation but may also increase the phagocytic potential of these cells. We have also successfully isolated from human skin dermal microvascular endothelial cells. These are the cells which are the targets of cutaneous or systemic necrotizing vasculitis, proliferate wildly in vascular tumors such as Kaposi's sarcoma and may be the earliest targets in skin graft rejection. Almost nothing is known about the immunological phenotype and immunological potential of these cells because of the extreme difficulty in isolating them. We have shown that these cells can be induced to develop HLA-DR antigen following exposure to gamma interferon. At least some of these cells also appear to possess receptors for the third component of complement. Endothelial cells derived either from umbilical veins or from the dermal microvasculature can be induced to differentiate in vitro on selective matrices and under certain culture conditions to form tube-like structures resembling blood vessels.

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. After treatment was stopped in order to determine the need for chronic therapy, one patient with the nevoid basal cell carcinoma syndrome developed more than 20 tumors within one year. The second patient with arsenic-induced tumors has not as yet developed new tumors as of the 6-month visit. The efficacy of isotretinoin as a chemopreventive agent is being studied further in a series of patients with xeroderma pigmentosum and nevoid basal cell carcinoma syndrome. Preliminary results indicate partial, but not complete, efficacy in inhibiting new tumor formation.

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. In contrast to the spinal ligament calcification induced by isotretinoin, predominantly extraspinal tendon and ligament calcification 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.

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. DNA, damaged by ionizing-radiation-induced free radicals, is considered to be the principal target for the effects of ionizing radiation. Damage to DNA arises also from free radicals resulting from normal intracellular metabolic reactions and we have postulated that such DNA damage may be a causative factor in cancer, aging, and the neurodegenerations of Alzheimer's disease and Parkinson's disease. Very few of the possible types of free-radical-induced DNA base damage have been identified in intracellular DNA. Those identified have required the radioactive labelling of DNA and have therefore been limited to the thymine moiety. We sought a technique for identifying base damage induced in intracellular DNA which does not require the use of radioactivity and which can unequivocably identify the many types of possible damage to each of the four DNA bases. Identification of such lesions induced in intracellular DNA would permit studies to determine their biological effects and whether they are subject to various DNA-repair processes in normal and patient (e.g., Alzheimer's disease) cells. The technique we have just successfully introduced for the study of intracellulary induced DNA damage is the gas chromatography-mass spectrometry (GC-MS) technique originally utilized by Dr. Miral Dizdaroglu for studying lesions induced in aqueous solutions of DNA and its precursors. In collaboration with Dr. Dizdaroglu, we have recently identified with the GC-MS technique the novel purine lesion 8 ,5'cyclo-2'-deoxyguanosine induced by ionizing radiation in the DNA of normal human lymphoblastoid lines. We are now planning studies to see if normal cells can

repair this particular type of DNA damage. Our success with the GC-MS technique indicates that it may well make possible the identification and study of many other lesions expected to be induced by ionizing radiation in intracellular DNA.

Chemistry, Structure, and Biosynthesis of Mammalian Epidermal Keratin Filaments:

We are continuing our studies of the physico-chemical structure of keratin and other type filaments. 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 complexes, arranged in an anti-parallel mode. In collaboration with Dr. A.C. Steven (Lab. of Phys. Biol., NIADDK) Dr. David Parry (Massey University) and Dr. R.D.B. Fraser (CSIRO Melbourne), this work has permitted the construction of "surface-lattice" models of filament structure in which the rod domains of the protein chains from 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 the precise alignment of the neighboring rod domains. However, the structural organization of the end domain is still uncertain. In the case of the keratins, we have now proposed that their glycine-serine-rich sequences interact by hydrophobic and H-bonding to form highly-folded and convoluted structures.

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 tachtoid 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 (Masey University, New Zealand) have permitted the construction of models on how this network may be assembled, and thus regulate gene expression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03657-12 D

PERIOD COVERED

October 1, 1985 to September 30, 1986

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: S. Shimada, Visiting Fellow, Dermatology Branch, DCBD, NCI
W. Caughman, Medical Staff Fellow, Dermatology Branch, DCBD, NCI
A. Gaspari, Medical Staff Fellow, Dermatology Branch, DCBD, NCI
C. Hauser, Guest Researcher, 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. of Immunology, NIAID

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.5	5.5	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 tissue. We have found that epidermal Langerhans cells are derived from precursors 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 promflammatory agent affecting other cells - especially neutrophils. During the past year 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 alpha and beta chain message for the T cell antigen receptor. Furthermore we have cultured these cells from nude mice and have found that they express the entire T cell antigen receptor on their membrane. These cells may therefore represent an extra thymic source of T cells in nude as well as normal mice.

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

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". These studies will hopefully result in our ability to generate primary in vitro responses to protein and tumor associated antigens.

We have found that epidermal Langerhans cells (in contrast to keratinocytes) express little or no major histocompatibility Class I antigen. The deficient expression of Class I antigens permitted highly purified Langerhans cell populations to be isolated from epidermal cell suspensions by treatment with anti Class I antibody and complement. It is likely that the uniquely low cell surface expression of Class I antigen by Langerhans cells has relevance to both the immune response in the skin as well as to mechanisms of skin allograft rejection.

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 rearranged genes of the alpha and beta chain for the T cell receptor. 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 cells may therefore serve as an extra thymic source of T cells in nude as well as normal mice.

Publications:

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Katz, S.I., Cooper, K.D., Iijima, M., Tsuchida, T.: The role of Langerhans cells in antigen presentation. J. Invest. Derm. 85: 96s-98s, 1985.

Katz, S.I.: The skin as an immunological organ. J. Amer. Acad. Dermatol. 13: 530-536, 1985.

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Fine, J.D., Breathnach, S.M., Fox, P.A., Neises, G.R., Stanley, J.R., Katz, S.I. Monclonal antibodies in dermatology: Studies with a unique cell-surface antigen defined specifically for keratinocytes by a monoclonal antibody. Am. J. Dermpath. 7: 171-179, 1985.

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Shimada, S., Schwartz, R.H., Katz, S.I.: Development and characterization of trinitrophenyl-specific L3T4$^+$ T-cell clones. J. Invest. Dermatol. in press, 1986.

Harvath, L., Yancey, K., Katz, S.I. Selective inhibition of human neutrophil chemotaxis to N-formyl-methionyl-leucyn-phenylalanine by sulfones. J. Immunol. in press, 1986.

Caughman, S.W., Breathnach, S.M., Sharrow, S.O., Stephany, D.A., Katz, S.I.: Culture and characterization of murine dendritic thy 1^+ epidermal cells. J. Invest. Derm. 86: 615-624, 1986.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03667-02 D

PERIOD COVERED
October 1, 1985 to September 30, 1986

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: Nurit Rubinstein, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI
Russell Eyre, M.D., Medical Staff Fellow, 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:
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.)*

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 also used antibodies to BP antigen, as well as to other basement membrane components, to rapidly diagnose various types of epidermolysis bullosa, often within the first few days after birth. We have demonstrated that autoantibodies from patients with fogo selvagem, a form of pemphigus endemic to Brazil, have similar specificities to autoantibodies from patients with sporadic North American PF. We have used autoantibodies from these bullous disease patients to help define stages of differentiation of keratinocytes. Specifically, the synthesis of BP and PV antigens are normally modulated, along with differentiation, by extracellular calcium concentration in culture. In virus sarcoma infected cells, this modulation goes awry. Finally, we are just beginning to try to characterize some of these antigens defined by these autoimmune diseases at the gene level.

GPO 914-918

Project Description

Major Findings:

Immunoprecipitation of extracts of cultured human keratinocytes indicates that almost all patients with bullous pemphigoid (BP) have autoantibodies against the same molecule, a polypeptide of molecular weight approximately 230,000.

A similar immunoprecipitation procedure indicates that patients with epidermolysis bullosa acquisita (EBA) have autoantibodies against a 290,000 dalton polypeptide, which is synthesized by both human keratinocytes and human dermal fibroblasts. A 145,000 dalton polypeptide that is detected by immunoblotting with EBA sera is not a precursor of the 290,000 dalton protein, and is probably a degradation product.

Autoantibodies from both BP and EBA patients that bind to the respective non-denatured antigens by immunoprecipitation may not bind to the antigens that have been denatured for immunoblotting.

A monoclonal antibody against desmoglein I, a core desmosomal glycoprotein, binds to pemphigus foliaceus (PF) antigen, defined by autoantibodies from a subgroup of PF patients. This finding provides further proof for the hypothesis that a subgroup of PF patients have autoantibodies against a desmosomal glycoprotein.

Sera from patients with fogo selvagem, a form of pemphigus endemic to Brazil, have similar antigenic specificities to sera from patients with sporadic North American or European PF.

Sarcoma virus infected keratinocytes do not differentiate normally, particularly in the modulation of synthesis of BP and pemphigus vulgaris antigen by calcium.

Epidermolysis bullosa simplex at birth may be severe and confused with the generally more severe types of epidermolysis bullosa that have a worse long-term prognosis. Immunofluorescence mapping studies performed with antibodies against basement membrane components may help diagnose these diseases.

Publications:

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Koulu, L., Stanley, J.R.: Clinical, histologic and immunopathologic comparison of pemphigus vulgaris and pemphigus foliaceus. Seminars in Dermatol. (in press).

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Stanley, J.R., Rubinstein, N., Klaus- Kovtun, V.: Epidermolysis bullosa acquisita antigen is synthesized by both human keratinocytes and human dermal fibroblasts. J. Invest. Dermatol. 85: 542-545, 1985.

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Butler, D.F., Berger, T.G., James, W.D., Smith, T.L., Stanley, J.R., Rodman, O.G.: Bart's syndrome: microscopic, ultrastructural and immunofluorescent mapping features. Pediatric Dermatol. 3: 113-118, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03666-08 D

PERIOD COVERED

October 1, 1985 to September 30, 1986

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
Yasuo Kubota, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI
Joseph Cason, Technician, Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

LCI, NIAID, Clinocal Hematology Branch, NHLBI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 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.)*

Antigen-antibody complexes and soluble mediators of inflammation such as the complement derived anaphylatoxins play important roles in a variety of human systemic and cutaneous diseases. Serum sickness is a prototypical immune complex disease. We have performed the first prospective indepth immunological and immunopathological study of serum sickness in man. We have defined the time course of the clinical, immunological and immunopathological findings in human serum sickness. We have described a hitherto unknown cutaneous sign in humans specific for serum sickness. Since immune complexes may activate the complement system and since the complement fragments C5a and C3a are thought to be important in the pathogenesis of the inflammatory response in cutaneous and systemic diseases, we have purified human C5a and C5a des Arg studied their in vivo and in vitro reactivity. In vivo role was assessed by the first in-depth analyses of the cutaneous reactivity of these complement fragments in man. We have also studied the ability of C5a and C3a to modulate cell surface receptors for immunoglobulin and complement on the surface of leukocytes. Increasing evidence indicates that human endothelial cells, under certain circumstances, can be induced to become immunologically competent. In order to evaluate the role endothelial cells in immune disorders of the skin we have isolated human umbilical vein, grown them in cell culture, examined them for the presence of immunologically relevant cell surface antigens and receptors before and after stimulation with soluble mediators of immunoregulation. In addition we have isolated human dermal microvascular endothelial cells from human foreskins, purified them successfully, passaged them in tissue culture and performed analyses of their immunological phenotypes.

Project Description

Major Findings:

Patients with the blistering skin disease, dermatitis herpetiformis have IgA deposits in their skin which are exclusively IgA_1. Circulating immune complexes contain both IgA_1 and IgA_2.

Human C5a has been purified to homogeneity while retaining full biologic activity. In vitro studies have shown C5a to be a potent chemotactic agent, to degranulate polymorphonuclear neutrophils and aggregate platelets.

Human C5a is a potent meditor of cutaneous inflammation. It causes the rapid onset of a wheal and flare reaction when injected into normal skin and the accumulation of polymorphonuclear neutrophils around the blood vessels in the superficial and mid dermis.

C5a causes the induction of Fc IgG, C3b and iC3b receptors on human peripheral blood monocytes and polymorphonuclear neutrophils in a dose dependent fashion.

The immediate wheal and flare response to human C5a is mediated in part by circulating blood elements. In vivo studies in patients with bone marrow failure anemia show that these patients have decreased wheal and flare reactivity to C5a but not to histamine or morphine. The decreased responsiveness to C5a is proportional to the extent of leukopenia and thrombocytopenia.

Patients with aplastic anemia were treated with infusions of horse antithymocyte globulin and monitored, in a prospective fashion, for the clinical, immunological and immunopathological signs and symptoms of serum sickness. Almost all patients studied developed signs and symptoms of serum sickness. These included fever, malaise, cutaneous eruptions, arthralgias, G-I upset, proteinuria and lymphadenopathy.

Most patients who develop serum sickness also develop a unique cutaneous sign, thought to be specific for serum sickness. This cutaneous sign, which has not been previously described, consists of a band of erythema along the sides of the hands, feet, fingers and toes. It is often the earliest sign of serum sickness.

Lesional skin from most patients with serum sickness shows deposits of IgM, IgA, IgE and C3 in the walls of small cutaneous blood vessels.

Human umbilical vein endothelial cells do not possess receptors for Fc IgG or C3. Infection with herpes simplex virus will induce Fc IgG, C3b and iC3b receptors but not C3d or Fc IgM receptors on endothelial cells.

Human dermal microvascular endothelial cells can be isolated from foreskins and purified by Percoll density gradient centrifugation. These cells can be induced to express HLA-Dr antigens by treatment with γ interferon.

Publications:

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Brickman, C.M., Tsokos, G.C., Chused, T.M., Balow, J.E., Lawley, T.J., Santaella, M., Hammer, C.H., Frank, M.M.: Immunoregulatory disorders associated with hereditary angioedema: 2. Serologic and cellular abnormalities. J. Aller. Clin. Immunol. (in press).

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Bielory, L., Gascon, P., Lawley, T.J., Nienhuis, A., Frank, M.M., Young, N.S.: Serum sickness and hematopoietic recovery from antithymocyte globulin in bone marrow failure patients. Br. J. Hematol. 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03659-12 D

PERIOD COVERED

October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 chererters 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 professionel personnel below the Principal Investigetor.) (Name, title, laboretory, 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
G. Gantt, Registered Nurse, Dermatology Branch, DCBD, NCI
K. Kraemer, Senior Investigator, Cell Genetics Br., NCI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesa, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.9	2.9	

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 spece provided.)*

Oral isotretinoin was effective in the prevention of skin cancer and in the treatment of a variety of disorders of kertinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne. Oral etretinate was more effective and less toxic than isotretinoin in the treatment of the disorders of keratinization. A high initial dose followed by a low maintenance dose of isotretinoin was comparably effective but less toxic than previously used continuous high-dosage schedules in the treatment of cystic acne. Psychological testing revealed decreased anxiety and depression in cystic acne patients after treatment with isotretinoin. Isotretinoin and etretinate treatment led to small but significant elevations in plasma lipids and changes in lipoproteins, which were responsive to dietary management. Absorption of etretinate is greater with milk, as a source of long-chain fatty acids, than with water. Etretinate is stored in fat and persists in the serum after discontinuation of therapy; trace amounts have been detected after more than 3 years. One chronic toxicity, "retinoid hyperostosis," characterized by anterior spinal ligament calcification and osteophyte formation of vertebrae, has been observed in 80% of patients treated with long-term, high-dose isotretinoin. Sixty percent of acne patients with moderate doses of isotretinoin for 9 months also developed vertebral osteophytes. Seven patients with xeroderma pigmentosum were entered into a cancer chemo-prevention study using isotretinoin. Of 5 patients entered into a phase I fenretinide-cancer chemoprevention study, 3 were discontinued due to toxicity (drug reaction, night blindness). Predominantly extraspinal tendon and ligament calcification 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.

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 more than 20 tumors within one year. The second patient with arsenic-induced tumors has not as yet developed new tumors as of the 6-month visit. The efficacy of isotretinoin as a chemopreventive agent is being studied further in a series of patients with xeroderma pigmentosum and nevoid basal cell carcinoma syndrome. Preliminary results indicate partial, but not complete, efficacy in inhibiting new tumor formation.

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.

In contrast to the spinal ligament calcification induced by isotretinoin, predominantly extraspinal tendon and ligament calcification 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.

Five patients with multiple basal cell carcinomas were treated with fenretinide, another new synthetic retinoid, for chemoprevention of new tumor formation. However, two patients had night blindness with abnormal dark adaptation tests and abnormal electroretinograms. This evidence of abnormal rod photoreceptor function was rapidly reversible on cessation of therapy. Possibly, fenretinide may interfere with the binding of vitamin A to opsin or with the transport of vitamin A. In addition, another patient developed a drug hypersensitivity reaction with an erythema multiforme-like rash, diarrhea and low-grade elevations of liver function tests. She recovered completely within one week after the drug was stopped.

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 self-consciousness.

Of 72 patients with cystic acne who were participating in isotretinoin treatment protocols and had a history of minocycline therapy, 4 were found to have apparently permanent tooth discoloration. No other cause was found for this tooth discoloration which in all likelihood is due to minocycline.

Publications:

Peck, G.L.: Therapy and prevention of skin cancer, in New Trends in Research and Therapy, Ed. J.H. Saurat, Karger, Basel, 1985, pp. 345-354.

Peck, G.L.: Retinoids and cancer. J. Invest. Dermatol. 85: 87-88, 1985.

DiGiovanna, J.J., Peck, G.L.: Vitamin A and the Retinoids, in Nutrition and the Skin, D. Roe (ed). Liss, New York, 1986, pp. 45-62.

Rubinow, D.R., Peck, G.L., Squillace, K.M., Gantt, G.: Reduced anxiety and depression in cystic acne patients after successful treatment with oral isotretinoin. J. Am. Med. Assoc. (in press).

Poliak, S.C., DiGiovanna, J.J., Gross, E.G., Gantt, G., Peck, G.L.: Minocycline-associated tooth discoloration in young adults. J. Am. Med. Assoc. 254: 2930-2932, 1985.

Kaiser-Kupfer, M.I., Peck, G.L., Caruso, R.C, Jaffe, M.J., DiGiovanna, J.J., Gross, E.G.: Abnormal retinal function associated with fenretinide, a synthetic retinoid. Arch. Ophthalmol. 104: 69-70, 1986.

Peck, G.L., DiGiovanna, J.J.: Retinoids, in Dermatology in General Medicine, 3rd edition, T.B. Fitzpatrick et al (eds). McGraw-Hill, New York, 1986, pp. 2582-2608.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03630-16 D

PERIOD COVERED: October 1, 1985 to September 30, 1986

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
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 B

☐ (a2) Interviews

SUMMARY OF WORK (Use standerd unreduced type. Do not excaed the space providad.)

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 limbskin.

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.

GPO 914-916

PHS 6040 (Rev. 1/84)

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 individial 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, psorisis, 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, hyperproliferatvie disorders of the epidermis exhibit inappropriate expression of proliferation-asociated keratin genes in the more superficial layers of the epidermis. In addition, skin cancers fail to expresses differentiation-associated keratins, indicative of their undifferentiated state.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03638-17 D

PERIOD COVERED

October 1, 1985 to September 30, 1986

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)*

Biostatistics Branch, DCCP, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.1	2.5	3.6

CHECK APPROPRIATE BOX(ES)

☒ (a) Human 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. Most studies have been conducted with cells from patients with xeroderma pigmentosum who have defective DNA repair plus multiple cutaneous malignancies and premature aging of sun-exposed skin and of the nervous system. Cells from patients with diseases with abnormal cell growth and differentiation, and with primary neuronal, muscular, and retinal degenerations are also being studied. These diseases include ataxia telangiectasia, Alzheimer disease, Parkinson disease, Huntington disease, Duchenne muscular dystrophy, retinitis pigmentosa, and Cockayne syndrome. These studies are designed to elucidate the pathogenesis of these disorders. We assess the biological effectiveness of DNA repair by 1) in vitro assays of cell survival after treatment of the cells with DNA-damaging agents; 2) analysis of chromosome aberrations in irradiated cells; and 3) transfection studies using irradiated shuttle vector plasmids. We search for DNA damage by 1) extracting the DNA and having it subjected to analysis by capillary-gas-chromatography mass-spectrometry; 2) studying unscheduled DNA synthesis induced in cultured cells by chemical carcinogens; and 3) determining the alkaline elution profile of DNA from cultured cells after their treatment with DNA-damaging agents.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Other Professional Personnel:

M.L. Dirksen, Ph.D., Expert, Dermatology Branch, DCBD, NCI
R.E. Tarone, Methematical Statistician, B, NCI
K.H. Kraemer, M.D., Senior Investigator, CH, NCI
K.W. Liao, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI
H. Jiang, M.D., Visiting Fellow, Drmatology Branch, DCBD, NCI
M.M. Dizdaroglu, Research Chemist, Ionizing Rad. Competency Program, NBS
J. Nove, Research Specialist, Laboratory of Radiobiology, Harvard School of Public Health
T. Kinsella, M.D., Senior Investigator, RO, NCI

Major Findings:

Hypersensitivity to the lethal effects of x-rays has been demonstrated in cultured lymphoblastoid cell lines from patients with the following neurodegenerations: Alzheimer disease, Parkinson disease, and Down syndrome. These studies have led to the concept that the sporadic forms of Alzheimer and Parkinson diseases are due to a somatic mutation early in embryogenesis.

Hypersensitivity to the lethal effects of the direct acting alkylating chemical N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) has been demonstrated in fibroblast strains from patients with Alzheimer disease.

We are collaborating with John Nove, Laboratory of Radiobiology, Harvard School of Public Health, in survival studies of fibroblast strains after their treatment with x-rays or tritiated water. We have found hypersensitivity in Usher syndrome and some Huntington disease strains. Duchenne muscular dystrophy strains were not hypersensitive.

We have found that the number of chromosome aberrations induced by ultraviolet radiation in Cockayne syndrome lymphoblastoid lines is the same as the number induced in xeroderma pigmentosum lines. Since patients with Cockayne syndrome do not develop sunlight-induced skin cancers, while xeroderma pigmentosum patients do, our results indicate that the number of induced chromosome aberrations per se cannot account for the large number of sunlight-induced skin cancers in xeroderma pigmentosum.

In collaboration with Miral Dizdaroglu, Ionizing Radiation Competency Program, National Bureau of Standards, we have applied capillary-gas-chromatography mass-spectrometry to identify damage induced by ionizing radiation in intracellular DNA. We have found the novel purine lesion 8,5'-cyclo-2'-deoxyguanosine in normal lymphoblastoid lines treated with ionizing radiation.

In collaboration with Timothy Kinsella, C RO, we have studied the alkaline elution patterns of DNA from Alzheimer disease fibroblasts after their treatment

with the alkylating chemical methylmethane sulfonate (MMS). In contrast to reports in the literature, our results show that the Alzheimer cells repair this chemical's DNA damage as well as normal cells.

In collaboration with Kenneth Kraemer, C CH, we are performing transfection of lymphoblastoid lines with shuttle vector plasmids which enable us to measure the inactivation of genes by ultraviolet radiation. We have shown that the test system readily detects the inability of xeroderma pigmentosum lines to repair the ultraviolet-irradiated plasmid.

Publications:

Robbins, J.H., Otsuka, F., Tarone, R.E., Polinsky, R.J., Brumback, R.A., and Nee, L.E.: Parkinson disease and Alzheimer disease: Hypersensitivity to X-rays in cultured cell lines. J. Neurol. Neurosurg. and Psychiatry. 48: 916-923, 1985.

Otsuka, F., Tarone, R.E., Seguin, L.R., and Robbins, J.H.: Hypersensitivity to ionizing radiation in cultured cells from Down syndrome patients. J. Neurol . Sci. 69: 103-112, 1985.

Robbins, J.H., Brumback, R.A., Polinsky, R.J., Wirtschafter, J.D., Tarone, R.E., Scudiero, D.A., Otsuka, F.: Hypersensitivity to DNA-damaging agents in abiotrophies: a new explanation for degeneration of neurons, photoreceptors, and muscle in Alzheimer, Parkinson and Huntington diseases, retinitis pigmentosa, and Duchenne muscular dystrophy. In Molecular Basis of Aging, Basic Life Science Series, Edited by A.D. Woodhead, A.D. Blackett, V. Pond and A. Hollander, Plenum Press, pp. 315-344, 1985.

Scudiero, D.A., Polinsky, R.J., Brumback, R.A., Tarone, R.E., Nee, L.E., and Robbins, J.H.: Alzheimer disease fibroblasts are hypersensitive to the lethal effects of a DNA-damaging chemical. Mutation Res. 159: 125-131, 1986.

Osuka, F., Robbins, J.H.: The Cockayne syndrome: an inherited multisystem disorder with cutaneous photosensitivity and defective repair of DNA. Comparison with xeroderma pigmentosum. Am. J. Dermatopathology. 7: 387-392, 1985.

Leech, R.W., Brumback, R.A., Miller, R.H., Otsuka, F., Tarone, R.E., and Robbins, J.H.: Cockayne syndrome: Clinicopathologic and tissue culture studies of affected siblings. J. Neuropathol. Exptl. Neurol. 44: 507-519, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03656-13 D

PERIOD COVERED
October 1, 1985 to September 30, 1986

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, Visiting Scientist, 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:
6.75	5.25	1.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The biosynthesis, structure and function of the protein chains which comprise the subunits of the keratin intermediate filaments of normal human, murine and bovine epidermis are being investigated. The subunits polymerize in vitro into native-type filaments. The details of filament ultrastructure are being investigated using image analysis procedures of filaments examined by transmission electron microscopic and scanning transmission electron microscopic techniques. Model structures generated from these methods will be computationally tested for compatibility with other physico-chemical data and amino acid sequence studies of individual filament subunits. cDNA cloned probes that encode human and mouse epidermal keratins have been isolated and are being used to determine the amino acid sequences of the proteins, and to study the structure and expression of keratin genes. A histidine-rich basic protein, termed filaggrin, isolated from human epidermis and the slightly different protein of mouse epidermis, specifically aggregate keratin filaments in a manner suggestive of an interfilamentous matrix component. cDNA cloned probes will be isolated to study their structure, expression and amino acid sequence of filaggrin.

GPO 914-918

Project Description

Other Professional Personnel:

P.E. Bowden, Ph.D., Visiting Associate, Dermatology Branch, DCBD, NCI
J.A. Rothnagel, Ph.D., Visiting Fellow, Dermatology Branch, DCBD, NCI
A. Blanchard, Ph.D., Visiting Fellow, 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., NIDDK
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

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 complexes, arranged in an antiparallel mode. In collaboration with Dr. A.C. Steven (Lab. of Phys. Biol., NIADDK) Dr. David Parry (Massey University) and Dr. R.D.B. Fraser (CSIRO Melbourne), this work has permitted the construction of "surface-lattice" models of filament structure in which the rod domains of the protein chains from 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. However, the structural organization of the end domain is still uncertain. In the case of the keratins, we have now proposed that their glycine-serine-rich sequences interact by hydrophobic and H-bonding to form highly-folded and convoluted structures.

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 tachtoid 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 (Massey University, New Zealand) have permitted the construction of models on how this network may be assembled, and thus regulate gene expression.

cDNA clones for mouse epidermal filaggrin have now been isolated by Dr. Rothnagel from libraries constructed from size-fractionated RNA, and sequenced. The largest clone isolated encoded about 300 amino acids, of which about 248 are unique and 50 represented the next repeat of the poly-protein. The clones will

be used to isolate the entire filaggrin gene from a human cosmid library in order to examine its gene structure, complexity and arrangement of repeats. In situ hybridization analyses reveal that filaggrin mRNA is precisely located over the granular layer of normal epidermis. This technology can now be adapted to examination of the expression and regulation of expression of filaggrin in normal and abnormally keratinizing human epidermis.

Dr. Bowden and Dr. Blanchard have now isolated a large number of human cosmid clones containing many different keratin genes. 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 appear to be several copies of the 67 kd "K1" keratin gene, one of which was previously isolated by this laboratory. Thus questions relating to the function of these multiple gene copies may now be resolved. In addition, regulatory sequences that permit the expression of this gene during terminal differentiation can be identified. Specific antibodies to the carboxyl-terminal end of the K1 protein have been produced, and the cDNA clones have been subcloned for in situ hybridization experiments. Accordingly, experiments on the expression of this gene during normal and abnormal keratinization can now be initiated. In related experiments in collaboration with Dr. Dennis Roop (LCTPP, DCE), the keratin genes have been transfected into cells for examination of the regulatory sequences. Further experiments using transfection into mouse oocytes, to generate transgenic mice containing the human keratin genes are in progress to study regulation.

Publications:

Steven, A.C., Jones, B.L., Hainfeld, J.F., Well, J.S., and Steinert, P.M.: Conformity and diversity in the structures of intermediate filaments. Ann. N.Y. Acad. Sci. 455: 371-380, 1985.

Steinert, P.M., Idler, W.W., Zhou, X.-M., Johnson, L.D., Parry, D.A.D., Steven, A.C., and Roop, D.R.: Structural and functional implications of amino acid sequences of keratin intermediate subunits. Ann. N.Y. Acad. Sci. 455: 451-461, 1985.

Steinert, P.M., and Steven, A.C.: Splitting hairs and other intermediate filaments. Nature, 316: 767, 1985.

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Goldman, A.E., Maul, G., Steinert, P.M., Goldman, R.D.: The keratin-like proteins which co-isolate with intermediate filaments of BH4.21 cells are unclear lamins. Proc. Natl. Acad. Sci., U.S. 83, in press, 1986.

Steinert, P.M.: Structural implications of the primary sequence of keratin and other intermediate filaments: In Processes in Cutaneous Epidermal Differentiation (Bernstein, I.A., and Hirone, T., eds), Tokyo University Press, in the press, 1986

Parry, D.A.D., Conway, J.F., Steinert, P.M.: Structural studies in lamin: similarities and differences between lamin and intermediate filament proteins. Biochem J, in press, 1986.

Steinert, P.M.: A stochastic model for the structure of keratin intermediate filaments. Proceedings of the IV International Psoriasis Symposium, Stanford University Press, in press, 1986.

Steinert, P.M. and Roop, D.R.: Intermediate filament genes in Intermediate Filaments, ed. by R.D. Goldman and P.M. Steinert, Plenum, New York, in press, 1986.

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1985 through September 30, 1986

The clinical research program of the Metabolism Branch is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations are carried out in patients with immunodeficiency diseases and a high incidence of neoplasia, as well as in patients with malignancy, especially T- and B-cell leukemias. These studies are directed toward defining the factors involved in the control of the human immune response. Major efforts in this area are directed toward: 1) studies of the arrangement of immunoglobulin genes and the 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 new transforming genes that translocate into the immunoglobulin gene locus in certain B-cell neoplasms; 2) somatic gene therapy from human genetic immunodeficiency diseases; 3) the genetic control of the immune response, especially as related to immune response genes associated with the major histocompatibility complex; 4) identification, purification, and molecular genetic analysis of unique cell surface determinants, especially receptors for growth factors on subpopulations of lymphoid cells with different functional capabilities using antibodies developed with hybridoma technology; 5) 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; 6) studies of the immune response including generation of specific antibodies and cytotoxic cells to viruses; and 7) the isolation and characterization of lymphokines 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 factilitate the development of techniques for the study of cell membranes, homeostatic mechanisms, and metabolic dearrangements of biochemical control mechanisms are being investigated. Within this area, special emphasis is placed on the biochemical events accompanying normal growth and hormonal control of this growth as it relates to our understanding of malignant growth and on the regulatory role on intermediary metabolism played by amino acids.

MOLECULAR ANALYSIS OF REARRANGEMENT OF IMMUNOGLOBULIN GENES AND T-CELL ANTIGEN RECEPTOR GENES: CLONAL AND LINEAGE MARKERS OF VALUE IN DIAGNOSIS OF LYMPHOID NEOPLASIA.

Previously, Drs. Korsmeyer and Waldmann used recombinant DNA technology involving analysis of Ig gene rearrangements to classify neoplasms previously of uncertain lineage, to aid in the diagnosis of neoplasms of the B-cell series and to define the state of differentiation of neoplastic B-cell precursors.

Over the past year, Dr. Waldmann has turned to a molecular genetic analysis of the genes encoding the T-cell antigen receptor to provide an analogous marker for T-cell clonality and lineage. The T-cell lymphocyte antigen specific receptor is a 90 kd heterodimer consisting of 40-50 kd Tα and Tβ subunits. Like immunoglobulins genes, the genes encoding the subunits undergo somatic rearrangements in the course of lymphocyte ontogeny. A third T-cell gene termed tγ has been identified which has many properties in common with Tα and T_β genes including assembly from gene segments resembling variable, joining and constant regions and rearrangements and expression in T-cells. The T-cell, alpha, beta and gamma receptor genes are located on chromosomes 14q11, 7q32-35 and 7p13-15 respectively. The T-cell lines and leukemias derived from patients with ataxia telangiectasia were shown to have a high incidence of chromosomal translocations and breaks at these sites. These patients appear to have a defect in DNA repair. Thus the site specific DNA breaks involved in the generation of active immunoglobulin and T-cell receptor genes may not be normally repaired in patients with ataxia telangiectasia an abnormality which may underlie the specific T-cell mediated and immunoglobulin immune response disorders of such patients. Dr. Waldmann has utilized probes to the genes encoding the alpha, beta and gamma chains of the T-cell antigen receptor and to immunoglobulin genes as a means to access clonality, lineage and state of maturation in lymphoid malignancies. A series of leukemias of B and T cells at various stages of maturation were analyzed for their receptor gene arrangement and expression into mRNA. All mature T cell leukemias studied rearranged Tγ and Tβ genes while J_H remained germline. The mature B cell leukemias studied rearranged J_H and either kappa or lambda light chain genes yet retained Tγ and Tβ genes in the germline configuration. These data suggest that the majority of mature leukemias have rearranged immunoglobulin or T cell receptor genes but not both. In contrast 5 of 9 B cells precursor leukemias and 1 of 4 pre T cells leukemias rearrange both J_H and T cell receptor genes. A number of factors may underlie this observation, first of all, the recombination signals for the T cell antigen receptor and for immunoglobulin heavy chain genes are similar and involve conserved 7 and 9 nucleotide elements separated by non-conserved spacer DNA that may be either 12 or 22 nucleotides long. Furthermore, a common recombinase appears to be involved in the variable region gene assembly events of both B and T cells. Furthermore, in B and T cells precursor leukemias the signals (the production of immunoglobulin molecules or T cell receptor molecules) that are present in more mature lymphocytes that terminate gene rearrangements are not expressed and thus there is no normal termination of recombinase activity. Using a combination of receptor gene rearrangments and mRNA expression the spectrum of leukemias studied within the T cell series permitted an assignment of the order of T cell gene rearrangements with Tγ gene rearrangement proceeding Tβ and Tβ rearrangements proceeding T_α expression. In these studies Southern blot analysis of clonally rearranged Tγ and β genes was sufficiently sensitive to detect even minority (2-5%) populations of clonal cells within tissues admixed with normal cellular elements thus providing an approach to the analysis of T cell clonality as an aid in the diagnosis of T cell malignancy, and in following the efficacy of therapy of T cell leukemias and lymphomas. There are few techniques available to define clonality in T-cell populations. Dr. Waldmann recently addressed the controversial issue concerning the clonality of the circulating lymphocytes in the T_γ lymphoproliferative disease a disorder characterized by an increase in the number of lymphocytes expressing the T3 and T8 antigens associated with granulocytopenia and anemia. In 8 of the 11 patients studied there was a

classical clonal pattern of Tβ and Tγ genes rearrangements. Thus in these patients the Tγ lymphocytosis associated with granulocytopenia and anemia appears to be an expansion of a single clone of lymphocytes with the T_3, T_8 positive phenotype.

Dr. Korsmeyer has demonstrated that many mature B-cell malignancies manifest chromosomal translocations that involve the immunoglobulin gene locus, that play a role in the malignant transformation and that are of value in the diagnosis and monitoring of the therapy of these malignancies. The t(14;18) (q32;q21) chromosomal translocation characteristic of over 80% of follicular B cell lymphomas is focused at the 5' end of immunoglobulin (Ig) joining (J_H) segments and was shown to have extranucleotide "N" segments at the site of the 14;18 juncture. This indicates that the immunoglobulin recombinase mediates breakage on chromosome 14 and suggests that the t(14;18) occurs early in development at a pre B cell stage. A cosmid clone of the germline region of 18q21 was obtained and a 2.8 kb major breakpoint region (mbr) where over 70% of translocations occur was sequenced and most breaks clustered within a 150 bp region. The breakpoint was shown to interupt an exon of a B cell associated gene at 18q21. This gene is expressed at high levels in pre B cells but down regulated with differentiation. In contrast the t(14;18) bearing mature B cell neoplasms have high levels of mRNA. The 18q21 gene is introduced head to tail with the Ig heavy chain locus and a fusion transcript containing J_H and 18q21 information is generated. cDNAs of the translocated and non-translocated species of the 18q21 gene have been obtained. The 18q21 major breakpoint region serves as a molecular marker for the translocation and thus a transformation event enabling Dr. Korsmeyer and his coworkers to follow the natural history of these neoplasms. A single translocation is invariant over time in each lymphoma. However, variation in Ig gene arrangement on the normal chromosome 14 and of light chain genes occur and are a secondary clonal variation representing clonal progression rather than true biclonality. Dr. Korsmeyer has also used the 18q21 major breakpoint region to refine lymphoma cytogenetics. Many 14q+ lymphomas (8/11) in which the reciprocal partner was indeterminant by routine cytogenetics were shown to be t(14;18) by gene rearrangement. In addition, Dr. Korsmeyer noted that some J_H rearrangements within T cell neoplasms can be classic 14;18 translocations indicating that just as D/J Ig rearrangements can spill over into T cells so can B cell type translocations.

Dr. Korsmeyer had identified and cloned a kappa chain gene deleting element (kde) that eliminates k genes prior to λ light chain gene expression. In further studies Dr. Korsmeyer noted in 75% of instances following k deletion the kde lands in the intervening sequence and proximal to this is an aberrant attempt at V/J_k joining. Twenty-five percent of the time the kde specifically-combines directly with the rearrangement signals flanking a V_k gene. Dr. Korsmeyer cloned and sequenced the germline form of the kde but can identify only a very short open reading frame that is evolutionarily conserved with the mouse.

SOMATIC GENE THERAPY FOR HUMAN GENETIC DISEASE

The major thrust of work of Dr. Blaese has been directed towards the development of techniques and reagents for use in gene therapy in the treatment of human disease. The most likely initial candidate disease in which gene therapy would be employed is the form of severe combined immune deficiency

(SCID) caused by deficiency of the purine salvage enzyme adenosine deaminase (ADA). Dr. Blaese has established unique T lymphocyte cell lines from four patients with ADA deficiency using the T lymphotropic transforming virus HTLV-I. Since the primary site of pathology in ADA deficiency is the T cell, these lines have provided us with an exceptionally valuable resource for learning more about the fundamental biochemical defect of this disease as well as giving us an in vitro test system for evaluating the efficiency of gene transfer and for the expression of the transfered genes leading to functional reconstitution of the cells. The level of ADA in each line was less than 1% of normal. Furthermore, the growth of each line was inhibited by deoxyadenosine at a concentration 100 fold less than that needed to inhibit the growth of normal T cell lines.

In collaboration with Drs. French Anderson of the NHLBI and Eli Gilboa at Princeton University, Dr. Blaese has been using these ADA deficient T cell lines to study retroviral mediated transfer of the human ADA gene. A murine retrovirus has been modified by the removal of its GAG, POL, and ENV coding sequences and the insertion of a bacterial neomycin resistance gene the human ADA cDNA and the promoter from an SV40 early region promoter. The ADA deficient T cells were infected with this ADA vector (called SAX) under various experimental protocols and then analyzed for ADA protein levels and deoxyadenosine resistence. The average level of gene transfer with this vector is 30% as measured by DNA dot blotting of individual T cell clones derived immediately following treatment of the ADA deficient T cell lines with the SAX vector. ADA levels in the vector DNA positive cells become normal as does the resistence to the toxic effects of deoxyadenosine. Over 90% of the clones containing at least a single copy of the vector produced functional human ADA at normal levels. Therefore this gene delivery system is very effective in transferring genes to human T lymphocytes and the transferred genes are expressed very efficiently holding promise that gene therapy for ADA deficiency may be possible within the not to distant future.

Towards this goal Dr. Blaese and coworkers have begun collaborative studies with Drs. Richard O'Reilly at the Memorial Sloan Kettering Cancer Center in New York and with Dr. Arthur Neinhius at the NHLBI using a bone marrow transplantation protocol in monkeys to test the gene transfer system. These studies, although still preliminary, have deomonstrated that one can successfully induce the production of some human ADA in SAX vector treated monkeys again demonstrating the potential for this mode of gene transfer.

THE GENETIC CONTROL OF THE IMMUNE RESPONSE.

A primary goal of Dr. Berzofsky's group has been to understand and ultimately manipulate the immune response at an antigen-specific level. To do so, they have been studying the mechanisms of immune response (Ir) gene function and T cell activation by antigen in association with major histocompatibility (MHC) antigens. In contrast to antibodies, the cellular immune response, they have found, is dominated by T cells specific for a few immunodominant antigenic sites. The immunodominance is due to a focusing of a polyclonal response onto a single region of the molecule, rather than to the preemption of the response by a single dominant clone. Dr. Berzofsky has shown that immunodominance of a site depends both on factors intrinsic to the antigen, such as helical amphipathicity (the property of having one side hydrophobic

and the other hydrophilic--see below), and on factors extrinsic to the antigen, especially the MHC antigens of the responding individual, and thus its Ir genes. In collaboration with Dr. Charles DeLisi and his group, Dr. Berzofsky has found that the majority of known immunodominant sites recognized by helper T cells have a common property, namely that if they fold into a helix, that helix would be amphipathic. They have developed a computer algorithm to predict such sites from the protein sequence alone, which correctly identifies 18 of 23 known immunodominant T cell sites on 12 proteins. The ability to predict T cell antigenic sites is being applied by Dr. Berzofsky's group for development of synthetic vaccines, to the AIDS virus and the malaria parasite.

Dr. Berzofsky's group, in collaboration with that of Dr. Scott Durum, discovered that helper T cell clones, on stimulation with antigen and presenting cells, induce IL-1 secretion by macrophages. This induction is mediated by a new low-molecular weight lymphokine secreted by the T cell clones, which is now being characterized. Dr. Berzofsky's group has also demonstrated a new population of IL-2-receptor-bearing T cells in unstimulated spleen cells, exclusively in the $L3T4^+$ $Lyt2^-$ subpopulation, and genetically regulated by non-MHC genes, especially one mapped to chromosome 7 of the mouse.

THE INTERLEUKIN-2 RECEPTOR ON NORMAL AND MALIGNANT LYMPHOCYTES

The IL-2 -IL-2 receptor system plays a key role in the T-cell immune response. In the past IL-2 binding was reported to be restricted to the 55 kd Tac peptide. This view that only a single peptide is involved in the IL-2 receptor leads to a number of unresolved questions including; What is the basis for the major difference in affinity between high (10^{-9}M) and lower affinity (10^{-12}M) IL-2 receptors? What is the mechanisms by which the IL-2 signal is transduced to the cell nucleus? And how does IL-2 act on Tac non expressing cells (e.g. resting NK or LAK cells)? Dr. Waldmann and coworkers investigating this issue have demonstrated that the IL-2 receptor is a complex receptor with multiple peptides in addition to the one identified by anti-Tac. A leukemic T-cell line that binds IL-2 yet does not bind four different antibodies (including anti-Tac and 7G7) that react with the Tac peptide was identified. This cell line manifested 6,800 receptors per cell with an affinity of 14nN. On the bases of cross-linking studies using ^{125}I-IL-2 this IL-2 binding peptide was shown to be larger than the Tac peptide with an approximate mr of 75,000. When similar cross-linking studies were performed on human T cell lymphotrophic virus 1 (HTLV-1) induced T cell lines (e.g. Hut 102) that manifest both high and low affinity receptors IL-2 binding peptides of both 55 kd and 75 kd were demonstrated whereas cross-linking to a line manifesting low affinity receptors alone (e.g. MT 1) only 55 kd receptors were present. Anti-Tac antibody did not block the binding of ^{125}I-IL-2 to MLA 144 cells. However this antibody abolished the binding of ^{125}I-IL-2 not only to the Tac peptide on Hut 102 cells and normal lymphoblasts but also to the 75 kd IL-2 binding peptide suggesting that this latter peptide is associated with the Tac peptide to form the high affinity IL-2 receptor complex.

Over the past year, Dr. Greene has molecularly cloned and sequenced the 5' flanking region of the IL-2 receptor gene that governs expression. S1 nuclease and primer extension analyses have demonstrated the presence of two discrete transcription initiation sites and two promoters separated by 58 bases which are utilized in normal activated T cells. In contrast, a unique transcription

initiation site located further upstream has been identified in HTLV-I infected adult T cell leukemic T cells which are characterized by high level, constitutive expression of IL-2 receptors. Utilizing IL-2 receptor promoter-CAT constructs, DNA regulatory sequences responsive to activation signals have been identified as well as the possible presence of an enhancer like element. A secreted form of the IL-2 receptor has been prepared by removal of the region encoding the carboxyterminal 29 amino acids in the IL-2 receptor cDNA. Expression of this "anchor minus" IL-2 receptor cDNA resulted in the production of a normally processed but secreted form of the receptor which retained the capacity to bind IL-2 and anti-Tac.

In a series of experiments performed by Dr. Greene in collaboration with Dr. William Haseltine's laboratory, the transactivator (tat) gene from HTLV-II has been studied for its capacity to alter the expression of various cellular genes. Recombinant retroviral expression vectors containing the tat-II gene were constructed and used to introduce this gene into Jurkat T cells and Raji B cells after amphotropic packaging in psi AM cells. The resultant cell lines produced a functional tat-II protein that was capable of transactivating the HTLV-I LTR. In Jurkat cells containing the tat-II gene, evidence was obtained at the RNA and protein level for the activation of IL-2 receptor, IL-2, and class II major histocompatibility gene expression. These data suggest that HTLV-I and II mediated T cell transformation may involve tat induced alterations in cellular gene expression. The coinduced expression of the IL-2 and IL-2 receptor genes may mediate autocrine T cell proliferation early in the transformation process. The induction of class II MHC antigen expression may also be involved in alterations in T cell growth.

Dr. Waldmann and coworkers have initiated a clinical trial to evaluate the efficacy of intravenously administered anti-Tac monoclonal antibody in the treatment of patients with the adult T cell leukemia. None of the 5 patients treated suffered any untoward reactions and none produced antibodies to the mouse immunoglobulin or to the idiotype of anti-Tac monoclonal. Three of the patients with a very rapidly progressing form of ATL had a very transcient response. Two of the patients had a temporary, partial or complete remission following anti-Tac therapy. In one of these patients therapy was followed by 5 months remission as accessed by routine hemological tests, immunofluorescence analysis of circulating T cells and molecular genetic analysis of arrangement of the gene encoding the beta chain of the T cell antigen receptor. Following the 5 months remission the patient's disease relapsed but a new course of anti-Tac infusions was followed by the virtual disappearance of skin lesions and an over 80% reduction of the number of circulating leukemic cells. Two months subsequently leukemic cells were again demonstrable in the circulation. At this time although the leukemic cells remained Tac positive and bound anti-Tac in vivo the leukemia was no longer responsive to infusions of anti-Tac and the patient required chemotherapy. This patient may have had the smouldering form of ATL initially when he responded to anti-Tac therapy wherein the leukemic cells may still require IL-2 for there proliferation. Alternatively the clinical responses may have been mediated by host cytotoxic cells reacting with the tumor cells bearing the anti-Tac mouse immunoglobulin on their surface by such mechanisms as antibody dependents cytotoxicity. These therapeutic studies have been extended in vitro by examining the efficacy of toxin conjugated to anti-Tac to selectively inhibit protein synthesis and viablity of Tac positive ATL lines. The addition of anti-Tac antibody coupled to

Pseudomonas exotoxin inhibited protein synthesis by Tac expressing Hut 102 B2 cells but not that by the Tac negative T cell line molt 4 which does not express the Tac antigen. In collaboration with Drs. FitzGerald and Pastan clinical trials of Pseudomonas exotoxin conjugated anti-Tac have been initiated. In further studies, Waldmann and coworkers showed that ^{212}Bismuth conjugated to anti-Tac using a bifunctional chelate eliminated greater than 98% of the proliferative capacity of the Tac expressing Hut 102 cells with only a modest effect on IL-2 receptor negative lines. This specific cytotoxicity was blocked by excess unlabelled anti-Tac but not by human IgG. Thus ^{212}Bi-anti-Tac is a potentially effective and specific immunocytotoxic agent for the elimination of IL-2 receptor positive cells.

Nelson and coworkers have demonstrated that in addition to cellular IL-2 receptor (IL-2R) activated normal peripheral blood mononuclear cells and certain lines of T and B cell origin release a soluble form of the IL-2R into the culture media. Using an enzyme-linked immunoabsorbent assay which employes two monoclonal antibodies that recognize distinct epitopes of the human IL-2R it was shown that normal individuals have measurable amount of IL-2R in their plasma and that certain lymphoreticular malignancies are associated with elevated plasma levels of this receptor. In addition to the secretion of IL-2R from activated normal T- and B-cells in vitro, Dr. Nelson observed that certain tumor cells constituatively secreted large amounts of IL-2R in vitro without prior activation. This was particularly true of tumor cells infected with human T-lymphotropic retrovirus type I (HTLV-I). In additional studies, Drs. Nelson and Rubin were able to show that the released soluble IL-2R was somewhat smaller (40-45 Kd) than the lymphocyte cell-surface IL-2R (50-55 Kd) and that the soluble form of the IL-2R, like the cell surface IL-2R was capable of binding to its ligand, IL-2. These in vitro studies suggested that the presence of IL-2R in solution might be a sensitive indicator of lymphocyte activation or the presence of various tumor cells. Elevated levels (mean 35,000; range 9,500-69,500 U/ml) of serum IL-2R were found in patients with the adult T-cell leukemia (ATL), a leukemia associated with HTLV-1 infection in humans. Levels of serum IL-2R comparable to that seen in patients with ATL were also found in the sera of patients with a leukemia of B-cells known as Hairy Cell Leukemia (HCL). Serum levels of IL-2R >2,000 U/ml were found in other patients with lymphoproliferative disorders including 3 of 7 patients with Hodgkins disease, 12 of 21 patients with the Sezary syndrome (a HTLV-I negative proliferation of mature T-cells), and 6 of 20 patients with chronic lymphocytic leukemia (a mature leukemia involving T-cells in some patients and B-cells in others). In patients with ATL and HCL, favorable clinical responses to therapy (including anti-Tac therapy in ATL and recombinant alpha interferon in HCL) were associated with reductions in serum IL-2R levels which rose again with disease relapses. These results suggest that serum levels of IL-2R may be useful in the diagnosis of various malignancies, monitoring patients for responses to therapy, and surveillence for early relapse following therapy. It was also interesting to observe that patients being treated with recombinant IL-2 for other malignancies, had >1000 fold rises in the levels of serum IL-2R following IL-2 therapy. In other studies designed to test whether the serum level of IL-2R at the time of diagnosis might be indicative of tumor burden and thus response to therapy, we analyzed the sera of 80 patients with non-Hodgkins lymphoma for IL-2R at the time of diagnosis and correlated this level with survival following chemotherapy. Patients with a serum IL-2R > 1000 U/ml had a 35% chance of

long term survival, while patients with levels < 1000 U/ml had an 85% chance of long term survival. In this regard, the level of serum IL-2R in these patients was the best prognostic test found to date for predicting survival at the time of diagnosis. Furthermore relative to homosexual and heterosexual controls, significantly elevated ($p<0.01$) levels of serum IL-2R were found in patients with the Acquired Immune Deficiency Syndrome (AIDS), those with HTLV-III associated Lymphadenopathy Syndrome (LAS), and those with the AIDS-related Complex (ARC).

Dr. Nelson and coworkers have also demonstrated that mouse fibroblasts transfected with a single full length IL-2R cDNA produce not only cell-associated IL-2R but also soluble IL-2R. Thus a single cDNA can encode both the soluble and cell-associated forms of the IL-2R.

ISOLATION AND CHARACTERIZATION OF BIOLOGICAL MODIFIERS THAT REGULATE THE HUMAN IMMUNE RESPONSE.

Dr. Muchmore has been pursuing the biochemistry, molecular biology and biologic importance of uromodulin an 85 kilodalton immunosuppressive glycoprotein originally isolated from human pregnancy urine. Uromodulin is a heavily glycosylated single chain protein composed of approximately 30% carbohydrate. Uromodulin is a high affinity ligand for both recombinant human IL-1 and recombinant human tumor necrosis factor. Interestingly, uromodulin is also a specific inhibitor of the activity of IL-1 in vitro. Biologic activity of uromodulin requires intact glycosylation. Dr. Muchmore has developed evidence that both IL-1 and TNF bind to uromodulin through exposed carbohydrate on the surface of uromodulin a partial amino acid sequence for uromodulin and a full length cDNA clone complete with an apparent leader sequence have been obtained.

MECHANISM OF ACTION OF INSULIN-LIKE GROWTH FACTORS.

The primary structure of insulin-like growth factors IGF I and II are closely related to insulin. In addition to an in vivo role for IGF-I as the mediator of pituitary growth hormone action, the IGFs are mitogens for a variety of cells in culture. There are two cell surface receptors for the IGFs. The type I receptor binds IGF-I better than IGF-II and recognizes insulin weakly. This receptor is a heterotetramer made up of alpha (M_r=130,000) and beta (M_r=95,000) subunits and thus is very similar to the insulin receptor. The type II receptor prefers IGF-II over IGF-I and does not recognize insulin; it is structurally quite distinct from the type I receptor being a single unit receptor, M_r=250,000. While the type I receptor has been shown to be important for growth stimulation by IGF in some cells, the role of the type II receptor has not been defined.

In order to sort out the respective roles of the type I and type II IGF receptors in mediating biologic responses, Dr. Nissley developed a blocking antibody to the type II receptor. The antibody does not block the binding of insulin to the insulin receptor nor does it block the binding of IGF-I to the type I receptor. Dr. Nissley has begun to utilize this anti-type II receptor antibody to investigate the role of the type II receptor in mediating biologic responses.

In the circulation IGFs are associated with "binding" or "carrier" proteins. There are two size classes of carrier proteins, a M_r=40,000 protein which is the only carrier protein in fetal blood and a M_r=150,000 protein which is under growth hormone control and found in postnatal blood. There are conflicting data regarding the structure of the 150 K binding protein. Some data suggest that the 150 K species is simply made up of six 25 K binding subunits whereas other data suggest that the 150 K carrier protein is made up of a 40-60 K binding subunit and a larger nonbinding subunit. To resolve these conflicting models Dr. Nissley has chemically crosslinked radiolabeled IGF-I and IGF-II to preparations of the 150 K binding protein and analysed the radioligand-binding protein complexes by sodium dodecylsulfate gel electrophoresis and autoradiography. His data suggest that the 48 K species generated from the 150 K binding protein is not simply a dimer of a 25 K species, contrary to the prediction of the 6 subunit model of the 150 K binding protein.

REGULATORY FUNCTIONS OF PROLINE AND ITS METABOLITE PYRROLINE-5-CARBOXYLATE

During the past year, Dr. Phang focused on the mechanisms mediating the effects of pyrroline-5-carboxylate (P5C) on cell regulation. This intermediate stimulates the early events in mitogenesis and acts synergistically with growth factors. Dr. Phang considered pyrroline-5-carboxylate reductase, which generates oxidizing potential accompanying the conversion of P5C to proline, as an important transducer. The enzyme has been purified by Dr. Phang. The subunits are 30 Kd on SDS PAGE and the native enzyme migrates as a 300 Kd decamer on HPLC.

The purified enzyme allowed Dr. Phang to attack a fundamental problem in the control of redox potential. P5C reductase can oxidize either NADH or NADPH. However, the effect of P5C on intact cells is limited to metabolic pathways coupled to the NADP/NADPH redox couple. To study the differential oxidation of pyridine nucleotides, he devised a method which simultaneously measures the oxidation of NADPH and NADH. Although NADH is oxidized as P5C concentrations are increased, this oxidation is inhibited by NADPH. The oxidation of NADPH, on the other hand, is insensitive to inhibition by NADH. Interestingly, the affinity of the enzyme for NADPH increases as P5C concentrations decrease which further tightens the interlock between P5C reduction and NADPH oxidation. Dr. Phang concludes that there may be distinct sites of oxidation for NADH and NADPH and the preferential oxidation of NADPH occurs at every concentration of P5C and NADH. Not only do these regulatory features help explain the stimulatory effects of P5C on intact cells, but also they provide an heuristic model for the family of oxireductases which influence the in situ redox state.

Dr. Phang has pursued metabolic studies in humans to consolidate his hypothesis that P5C can serve as an intercellular communicator. Although Dr. Phang previously showed that P5C is present in human plasma and increases in response to a load of dietary protein, he was uncertain as to the physiologic relevance of these perturbations. He monitored hourly plasma P5C levels in normal volunteers on a normal ad lib hospital diet. A striking diurnal pattern was observed. From midnight until 6 AM, the levels are relatively stable averaging about 0.2 uM. Three distinct peaks were seen during the day which roughly coincided with meals. Interestingly, each

sequential peak climbed to a higher level. The highest levels were obtained at about 9-10 PM and reached a level 10-15 X those seen in the early AM. These changes in P5C were observed in the face of relatively little change in plasma amino acids. When the volunteer was fasted for 24 hours, the P5C levels remained at the early AM level throughout the day. The increase in P5C levels on a normal diet are comparable to those seen for major regulatory hormones, e.g. insulin. Such responses are unique among amino acids. More importantly, the levels attained during these diurnal changes are adequate to produce agonist effects in intact cells in vitro. These observations argue for the physiologic relevance of Dr. Phang's in vitro findings and greatly strengthen the view that P5C functions as a diet-regulated intercellular communicator.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04002-17 MET

PERIOD COVERED

October 1, 1985 through September 30, 1986

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)*

Thomas A. Waldmann	Branch Chief	MET, NCI
Michael Davey	Medical Staff Fellow	MET, NCI
Robert Kozak	Guest Researcher	MET, NCI
Mitsura Tsudo	Visiting Fellow	MET, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

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

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.)*

The rearrangements of immunoglobulin and T cell antigen receptor genes were analyzed to develop approaches for the classification of neoplasms of uncertain lineage, to define the state of differentiation of neoplastic B-Cell and T-cell precursors, and to determine clonality of such lymphocytes. These studies have culminated in the development of new approaches for the diagnosis and monitoring the therapy of T-and B-cell lymphoid neoplasia. A hierarchy of immunoglobulin and T cell gene rearrangements was demonstrated. Immunoglobulin gene rearrangements progress from heavy chain rearrangements to light chain gene rearrangements and from κ light chain gene rearrangements to λ . T cell gene rearrangements were shown to progess from TCR γ to β to α gene rearrangements. Furthermore, studies were directed toward defining the role played by the inducible receptor for interleukin-2 (IL-2) in the proliferation of normal and malignant T cells. The high affinity IL-2 receptor was shown to be a complex of the 55 and 75 kd, IL-2 binding peptides. In contrast on normal resting T cell human T cell Lymphotrophic virus I-(HTLV-I) associated adult T-cell Leukemia (ATL) cells constituatively express large number of IL-2 receptors which may be aberrant. The anti-Tac monoclonal antibody was used in the therapy of the until now universally fatal Tac expressing ATL. three of six patients treated had a partial or complete temporary remission. In additional studies Pseudomonas exotoxin or ^{212} Bismuth were conjugated to the anti-Tac monoclonal antibody. These antibody conjugates effectively killed Tac expressing tumor lines at concentrations that did not effect the viability of Tac nonexpressing T-lymphocytes.

GPO 914-918

Project Description

Objectives:

The objectives of the study were: to use a molecular genetic analysis of the arrangement of immunoglobulin and T-cell receptor genes to classify neoplasms of uncertain lineage, to define the state of differentiation of lymphoid tumors, to determine the mechanisms that lead to the failure of maturation of such precursor cells, and to develop new approaches for the diagnosis and monitoring the therapy of lymphoid neoplasia. Other objectives were to purify and characterize the membrane receptor for IL-2, to clone and express the gene for this receptor, to study the expression of this receptor in varying lymphoid malignancies, to determine the effect of a monoclonal antibody (anti-Tac) to the IL-2 receptor on the human immune response in vitro, and to determine the efficacy of therapy with anti-Tac monoclonal, both unmodified and conjugated with Pseudomonas toxin, or ^{212} Bismuth in the treatment of patients with ATL.

Methods Employed:

Recombinant DNA techniques with ^{32}P-labeled probes to the constant region genes of the γ chain and β chain of the T-cell antigen receptor, to the γ chain of T cells,and to the constant κ , λ light chain and heavy chain V,D,J, and C chain genes of immunoglobulins were used to study antigen-specific T-cell receptor and immunoglobulin gene rearrangements that occur during T-cell and B-cell maturation and to analyze the arrangements of such genes in lymphoid leukemias and immunodeficiency states. Hybridoma procedures have been used to produce a monoclonal antibody to IL-2 receptors. This monoclonal antibody was used to purify the IL-2 receptor by affinity chromatography. Utilizing an oligonucleotide probe to the determined N terminal amino acid sequence of this receptor, cDNAs encoding the IL-2 receptor were cloned and expressed in COS-1 cells. Cross linking studies with ^{125}I-IL-2 were used to identify IL-2 binding peptides in leukemic T-cells lines including those with high and low affinity IL-2 receptors as well as those with low affinity receptors alone. In vitro culture techniques utilizing mitogen-stimulated peripheral blood lymphocytes have been developed for the study of the terminal differentiation of B lymphocytes into immunoglobulin-secreting cells and for the analysis of helper and suppressor T-cell and monocyte activity. These techniques have been applied to the analysis of helper and suppressor T-cell functions of leukemias, the analysis of the functional effects of a monoclonal antibody to the IL-2 receptor, and to the analysis, identification, and partial purification of a suppressor molecule that inhibits immunoglobulin synthesis but does not affect T-cell proliferation.

Major Findings:

Previously, Drs. Korsmeyer and Waldmann used recombinant DNA technology involving analysis of Ig gene rearrangements to classify neoplasms previously of uncertain lineage, to aid in the diagnosis of neoplasms of the B-cell series and to define the state of differentiation of neoplastic B-cell precursors. Over the past year, Dr. Waldmann has turned to a molecular genetic analysis of the genes encoding the T-cell antigen receptor to provide an analogous marker for T-cell clonality and lineage. The T-cell lymphocyte antigen specific receptor is a 90 kd heterodimer consisting of 40-50 kd Tα and Tβ subunits. Like immunoglobulin genes, the genes encoding these subunits undergo somatic rearrangements in the course of lymphocyte ontogeny. A third T-cell gene termed Tγ has been identified which has many properties in common with Tα and T_β genes including assembly from gene segments resembling variable, joining and constant regions and rearrangements and expression in T-cells. The T-cell alpha, beta and gamma receptor genes are located on chromosomes 14q11, 7q32-35 and 7p13-15 respectively. The T-cell lines and leukemias derived from patients with ataxia telangiectasia were shown to have a high incidence of chromosomal translocations and breaks at these sites. These patients appear to have a defect in DNA repair. Thus the site specific DNA breaks involved in the generation of active immunoglobulin and T-cell receptor genes may not be normally repaired in patients with ataxia telangiectasia, an abnormality which may underlie the specific T-cell mediated and immunoglobulin immune response disorders of such patients. Dr. Waldmann has utilized probes to the genes encoding the alpha, beta and gamma chains of the T-cell antigen receptor and to immunoglobulin genes as a means to assess clonality, lineage and state of maturation in lymphoid malignancies. A series of leukemias of B and T cells at various stages of maturation was analyzed for receptor gene arrangement and expression into mRNA. All mature T cell leukemias studied rearranged T_γ and T_β genes while J_H remained germline. The mature B cell leukemias studied rearranged J_H and either kappa or lambda light chain genes yet retained T_γ and T_β genes in the germline configuration. These data suggest that the majority of mature leukemias have rearranged immunoglobulin or T cell receptor genes but not both. In contrast 5 of 9 B cell precursor leukemias and 1 of 4 pre T cells leukemias rearrange both J_H and T cell receptor genes. A number of factors may underlie this observation. First of all, the recombination signals for the T cell antigen receptor and for immunoglobulin heavy chain genes are similar and involve conserved 7 and 9 nucleotide elements separated by non-conserved spacer DNA that may be either 12 or 22 nucleotides long. Furthermore, a common recombinase appears to be involved in the variable region gene assembly events of both B and T cells. Furthermore, in B and T cell precursor leukemias the signals (the production of immunoglobulin molecules or T cell receptor molecules) that are present in more mature lymphocytes that terminate gene rearrangements are not expressed and thus there is no normal termination of recombinase activity. Using a combination of receptor gene rearrangments and mRNA expression the spectrum of leukemias studied within the T cell series permitted an assignment of the order of T cell gene rearrangements with Tγ gene rearrangement preceding Tβ and Tβ rearrangements proceeding Tα expression. In these studies Southern blot analysis of clonally rearranged Tγ and β genes was sufficiently sensitive to detect even minority (2-5%) populations of clonal cells

within tissues admixed with normal cellular elements thus providing an approach to the analysis of T cell clonality as an aid in the diagnosis of T cell malignancy, and in following the efficacy of therapy of T cell leukemias and lymphomas. There are few techniques available to define clonality in T-cell populations. Dr. Waldmann recently addressed the controversial issue concerning the clonality of the circulating lymphocytes in the T_γ lymphoproliferative disease, a disorder characterized by an increase in the number of lymphocytes expressing the T3 and T8 antigens associated with granulocytopenia and anemia. In 8 of the 11 patients studied there was a classical clonal pattern of Tβ and Tγ gene rearrangements. Thus in these patients the Tγ lymphocytosis associated with granulocytopenia and anemia appears to be an expansion of a single clone of lymphocytes with the T_3, T_8 positive phenotype.

Studies on the structure, function and expression of the Interleukin-2 receptor were extended. The IL-2 receptor system plays a key role in the T-cell immune response. In the past IL-2 binding was reported to be restricted to the 55 kd Tac peptide. This view that only a single peptide is involved in the IL-2 receptor leads to a number of unresolved questions including: What is the basis for the major difference in affinity between high (10^{-12}M) and lower affinity (10^{-9}M) IL-2 receptors? What is the mechanism by which the IL-2 signal is transduced to the cell nucleus? And how does IL-2 act on Tac non-expressing cells (e.g. resting NK or LAK cells)? Dr. Waldmann and coworkers investigating this issue have demonstrated that the IL-2 receptor is a complex receptor with multiple peptides in addition to the one identified by anti-Tac. A leukemic T-cell line (MLA-144) that binds IL-2 yet does not bind four different antibodies (including anti-Tac and 7G7) that react with the Tac peptide was identified. This cell line manifested 6,800 receptors per cell with an affinity of 14nM. On the basis of cross-linking studies using ^{125}I-IL-2 this IL-2 binding peptide was shown to be larger than the Tac peptide with an approximate mr of 75,000. When similar cross-linking studies were performed on human T cell lymphotrophic virus 1 (HTLV-1) induced T cell lines (e.g. Hut 102) that manifest both high and low affinity receptors IL-2 binding peptides of both 55 kd and 75 kd were demonstrated. When cross-linking to a line manifesting low affinity receptors alone (e.g. MT 1) only 55 kd receptors were present. Anti-Tac antibody did not block the binding of ^{125}I-IL-2 to MLA 144 cells. However this antibody abolished the binding of ^{125}I-IL-2 not only to the Tac peptide on Hut 102 cells and normal lymphoblasts but also to the 75 kd IL-2 binding peptide suggesting that this latter peptide is associated with the Tac peptide to form the high affinity IL-2 receptor complex.

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following anti-Tac therapy. In one of these patients therapy was followed by a 5 month remission as assessed by routine hematological tests, immunofluorescence analysis of circulating T cells and molecular genetic analysis of arrangement of the gene encoding the beta chain of the T cell antigen receptor. Following the 5 month remission the patient's disease relapsed but a new course of antiTac infusions was followed by the virtual disappearance of skin lesions and an over 80% reduction of the number of circulating leukemic cells. Two months subsequently leukemic cells were again demonstrable in the circulation. At this time although the leukemic cells remained Tac positive and bound anti-Tac in vivo the leukemia was no longer responsive to infusions of anti-Tac and the patient required chemotherapy. This patient may have had the smoldering form of ATL initially when he responded to anti-Tac therapy wherein the leukemic cells may still require IL-2 for their proliferation. Alternatively the clinical responses may have been mediated by host cytotoxic cells reacting with the tumor cells bearing the anti-Tac mouse immunoglobulin on their surface by such mechanisms as antibody dependent cytotoxicity. These therapeutic studies have been extended in vitro by examining the efficacy of toxins conjugated to anti-Tac to selectively inhibit protein synthesis and viability of Tac positive ATL lines. The addition of anti-Tac antibody coupled to Pseudomonas exotoxin inhibited protein synthesis by Tac expressing Hut 102 B2 cells but not that by the T cell line MOLT 4 which does not express the Tac antigen. In collaboration with Drs. Fitzgerald and Pastan clinical trials of Pseudomonas exotoxin conjugated anti-Tac have been initiated. In further studies, Waldmann and coworkers showed that ^{212}Bismuth conjugated to anti-Tac using a bifunctional chelate eliminated greater than 98% of the proliferative capacity of the Tac expressing Hut 102 cells with only a modest effect on IL-2 receptor negative lines. This specific cytotoxicity was blocked by excess unlabelled anti-Tac but not by human IgG. Thus ^{212}Bi-anti-Tac is a potentially effective and specific immunocytotoxic agent for the elimination of IL-2 receptor positive cells.

Significance to Biomedical Research and the Program of the Institute:

The studies of leukemias using recombinant DNA technology are providing insights into the earliest events in B- and T-cell maturation and are aiding in the classification of maligancies that were previously of controversial origin. They are providing methods for the early diagnosis and the definition of the lineage of lymphoid malignancies and are providing insights into the pathogenesis of the disorders of maturation of malignant B and T lymphoid cells. Taken in conjuction with studies of transposed oncogenes these studies are providing insights into the nature of the malignant transformation itself. The studies related to the definition of the IL-2 receptor are important in our understanding of the mechanisms involved in the control of T-cell proliferation and in the differentiation of T cells into cytotoxic, suppressor, and helper T-cell populations. Furthermore, they are providing important new information concerning the pathogenesis of the uncontrolled growth of leukemic T cells of patients with HTLV-I associated T-cell leukemia. They have provided the scientific basis for a new approach to the therapy of human malignancy: the use of monoclonal antibodies to a growth factor receptor expressed on malignant but not on normal resting T cells. These studies are among the earliest to

utilize toxin conjugates of a monoclonal antibody in the treatment of human malignancy. In general these studies are providing the scientific basis for the development of rational strategies for the therapy of immunodeficiency autoimmune and diseases.

Proposed Course of Research:

The analysis of the IL-2 receptor on normal and malignant T cells will be continued. Special emphasis will be made on studies of the 75 kd IL-2 binding protein to define its structure, to produce monoclonal antibodies directed toward this peptide and to define whether it is associated with the 55 kd Tac IL-2 binding peptide in a complex IL-2 receptor. The distribution of IL-2 receptors on non-lymphoid cells will be analyzed following a preliminary observation that monocyte, granocytes and endothelial cells may manifest IL-2 receptors. The efficacy of anti-Tac monoclonal antibody and anti-Tac coupled to toxins or isotopes in the treatment of patients with Tac positive adult T-cell leukemia will be extended. Furthermore, a series of studies have been initiated to define the effect of anti-Tac in organ transplantation protocols. These later studies are based on observations that anti-Tac inhibits the mixed leukocyte reaction, the generation of cytotoxic T cells and that Tac positive cells are present in transplanted organs undergoing rejection. Furthermore, the efficacy of immunotherapy with anti-Tac coupled to Pseudomonas toxin will be studied in patients with Tac positive T-cells involved in autoimmune disorders. Specifically studies have been initiated on the effect of anti-Tac in the therapy of patients with aplastic anemia who have Tac positive cells that inhibit hematopoesis. In additional studies special emphasis will be placed on the analysis of the antigen specific T cell receptor genes and their rearrangement. We will use the analysis of the rearrangement of these genes to identify the lineage and clonality of neoplasms. Finally we will continue studies that have been initiated on putative transforming genes that are involve translocations into the sites of the T-cell receptor genes on chromosomes 14q11, 7p14 and 7q32.

Honors and Awards

1985 President's Lecture, American Society of Hematology
1985 Rufus Cole lecture in Medicine The Rockefeller University Hospital
1986 George W. Waterman lecture American Cancer Society Rhode Island Division
1986 Distinguished Lecture at combined program of American Society for Clinical Investigation and American Society for Clinical Research
1986 Socolow Lecture University of California San Francisco
1986 Selected as Honorary fellow American Academy of Allergy and Immunology
1986 Lila Gruber Cancer Research Award
The American Academy of Dermatology
1986 RGK Foundation lecture
U. Texas Health Science Center
1986 Alpha Omega Alpha Lecture
U. Penn. Medical School
1986 Leo Creep Lecture University of Pittsburg

Publications:

Greene, W.C., Depper, J.M., Crabtree, G.M., Kronke, M.J., Rudikoff, S., Robb, R.J., Waldmann, T.A., and Leonard, W.J.: Molecular cloning of the human IL-2 receptor. In Neth, R., and Gallo, R.C. (Eds.): Modern Trends in Human Leukemia, Springer Verlag, Berlin, 1985, pp. 385-395.

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Depper, J.M., Leonard, W.J., Kronke, M., Waldmann, T.A., and Greene, W.C.: The role of protein kinase C and effects of 5-azacytidine on regulation of interleukin-2 receptor expression. In: Cellular and Molecular Biology of Lymphokines (Sorg, P., and Schimpl, A., eds.), Academic Press, New York, pp. 151-156, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04004-24 MET

PERIOD COVERED

October 1, 1985 through September 30, 1986

TITLE OF PROJECT *(80 chererters 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 Investigetor.) (Name, title, laboratory, and institute affiliation)*

PI:	James M. Phang	Section Head	MET, NCI
	G. Alexander Fleming	Medical Staff Fellow	MET, NCI
	Patricia Cortazar	Guest Researcher	MET, NCI

COOPERATING UNITS *(if eny)*

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

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

The metabolism of proline and pyrroline-5-carboxylate (P5C) provides a mechanism for the intercompartmental, intercellular, and interorgan transfer of biologic information. The information is in the form of redox potential. (1) The transfer of redox potential by P5C provides a metabolic interlock between amino acids and ribonucleotides, an interlock which is modulated by growth factors as an early event during mitogenesis. The synergistic stimulation of PP-Rib-P synthesis by P5C and platelet-derived growth factor (PDGF) has been characterized in normal human fibroblasts. (2) P5C serves as an intercellular communicator by transferring biologic information from one organ to another. Plasma levels of P5C in humans are responsive to dietary intake and increase 10- to 15-fold with a normal diet as compared to the fasting state. Thus, P5C acts as a nutritionally sensitive "hormone" to transfer redox potential. (3) P5C reductase, the enzyme which catalyzes this transfer of redox potential, has been purified to homogeneity from human cells for the first time. On the basis of kinetics and regulatory mechanisms, it can be concluded that the enzyme functions primarily to catalyze the P5C-dependent oxidation of NADPH, thereby providing the mechanism for the metabolic interlock.

Project Description

Major Findings:

1. P5C Reductase

During the past year, we focused on the mechanisms mediating the effects of pyrroline-5-carboxylate (P5C) on cell regulation. This intermediate stimulates the early events in mitogenesis and acts synergistically with growth factors. We considered P5C reductase, which generates oxidizing potential accompanying the conversion of P5C to proline, as an important transducer. The enzyme has been purified from human cells for the first time. With human red cells as the source, we used 2',5'-ADP sepharose affinity chromatography followed by DEAE-sepharose ion-exchange chromatography and obtained homogeneous material. The subunits are 30 kDa on SDS-PAGE, and the native enzyme migrates as a 300 kDa decamer on HPLC. Enzyme activity is stable at -70 in the presence of 0.2 M KCl and 0.1 mM NADPH.

The purified enzyme allowed us to attach a fundamental problem in the control of redox potential. P5C reductase can oxidize either NADH or NADPH. However, the effect of P5C on intact cells is limited to metabolic pathways coupled to the NADP/NADPH redox couple. To study the differential oxidation of pyridine nucleotides, we devised a method which simultaneously measures the oxidation of NADPH and NADH. NADPH is labeled with tritium in the exchangeable position. The incorporation of tritium into proline concomitant to the conversion of 14C-P5C to 14C-proline allowed quantitation of the source of reducing potential utilized by the enzyme. With physiologic concentrations of NADH, NADPH, and P5C, the reduction of P5C to proline is accompanied solely by the oxidation of NADPH. Although NADH is oxidized as P5C concentrations are increased, this oxidation is inhibited by NADPH. The oxidation of NADPH, on the other hand, is insensitive to inhibition by NADH. We conclude that the preferential oxidation of NADPH occurs at physiologic concentration of P5C and NADH. Not only do these regulatory features help explain the stimulatory effects of P5C on intact cells, but they also provide an heuristic model for the family of oxio-reductases which influence the *in situ* redox state.

2. Synergistic Effects of P5C with Growth Factors

We previously showed that P5C markedly synergizes the stimulatory effect of PDGF on phosphoribosyl pyrophosphate production in quiescent cells. By contrast, insulin and the IGFs are additive rather than synergistic with P5C. The synergistic effect does not require protein synthesis. Although cycloheximide inhibited the effects of PDGF and IGFs on PRPP, it did not inhibit the synergism between PDGF and P5C. Although PDGF enhanced the effect of P5C on the production of ribose-5-phosphate by the pentose phosphate shunt, the effect of PDGF itself on PRPP does not require activation of the pentose phosphate shunt. We are currently studying the effect of growth factors on the activity of P5C reductase in various cell fractions to elucidate the mechanism of the P5C interaction with growth factors.

3. P5C Metabolism in Humans

We have pursued metabolic studies in humans to consolidate our hypothesis that P5C can serve as an intercellular communicator. Although we previously showed that P5C is present in human plasma and increases in response to a load of dietary protein, we were uncertain as to the physiologic relevance of these perturbations. With the use of a heparin lock, we monitored hourly plasma P5C levels in normal volunteers on a normal ad lib hospital diet. A striking diurnal pattern was observed. From midnight until 6 a.m., the levels are relatively stable, averaging about 0.2 μM. Three distinct peaks were seen during the day, which roughly coincided with meals. Interestingly, each sequential peak climbed to a higher level. The highest levels were obtained at about 9 to 10 p.m. and were 10 to 15 times those seen in the early a.m. These changes in P5C were observed in the face of relatively little change in plasma amino acids. When the volunteer was fasted for 24 hours, the P5C levels remained at the early a.m. level throughout the day. The increase in P5C levels on a normal diet are comparable to those seen for major regulatory hormones, e.g., insulin. Such responses are unique among amino acids. More importantly, the levels attained during these diurnal changes are adequate to produce agonist effects in intact cells in vitro. This intercellular communication may have the redox state in target tissues as an endpoint. In patients with Type 2 hyperprolinemia, an inborn error of metabolism with high concentrations of plasma P5C, the redox state becomes more oxidized. Lactate/pyruvate ratios are markedly altered in favor of pyruvate. We think that these observations argue for the physiologic relevance of our in vitro findings and support the hypothesis that P5C functions as a diet-regulated intercellular communicator.

Publications:

Fleming, G.A., Steel, G., Valle, D., Granger, A.S., and Phang, J.M.: The aqueous humor of rabbit contains high concentrations of pyrroline-5-carboxylate. Metabolism (in press).

Hegedorn, C.H., and Phang, J.M.: Catalytic transfer of hydride ions from NADPH to oxygen by the interconversions of proline and pyrroline-5-carboxylate. Arch. Biochem. Biophys. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04015-15 MET

PERIOD COVERED

October 1, 1985 through September 30, 1986

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

Development and Function of Humoral and Cellular Immune Mechanisms

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

PI:	R. Michael Blaese	Section Head	MET, NCI
	Andrew V. Muchmore	Senior Investigator	MET, NCI
	Basil Golding	Medical Staff Fellow	MET, NCI
	Don Kohn	Medical Staff Fellow	MET, NCI
	Alan Winkelstein	Guest Researcher	

COOPERATING UNITS *(if any)*

Mark Ballow	IPA	
French Anderson	Lab Chief	LMH, NHLBI

LAB/BRANCH

Metabolism Branch

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	5	2

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.)*

We have continued to work on several long-term projects and have initiated a major new area of research during the past year. Our long-term interest in the clinical effects of the Epstein-Barr virus (EBV) and defense mechanisms for its control have continued. EBV is a unique viral pathogen since it attacks the immune system itself and can result in transformation of infected B lymphocytes in vitro. It has been associated with B-cell lymphomas in immunocompromised patients. We have developed techniques for quantitating EBV-infected B cells and have been studying patients on intensive immunosuppression with cyclosporin A, a drug associated with the development of EBV-induced B-cell lymphomas. Our data show that we can now monitor patients for uncontrolled EBV-infected B-cell expansion. In a new area, we have begun a major collaborative effort with Dr. French Anderson of the NHLBI directed toward eventual gene therapy of the immune deficiency disease caused by a deficiency of the purine salvage enzyme, adenosine deaminase (ADA). We have established T-cell lines from ADA(-) patients using HTLV-I and have shown that these T-cell lines are sensitive to deoxyadenosine and are deficient in ADA, just as the cells respond in vivo. A mouse retrovirus vector gene transfer system has been modified to contain the gene for human ADA, and this vector has been tested for its ability to transfer a functional ADA gene into those ADA(-) T cells. Our data show that such a gene vector system can successfully transfer a functional human gene into defective cells with high efficiency and that such "gene"-treated cells are reconstituted to normal function in vitro.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Major Findings:

Our studies of the immunobiology of the Epstein-Barr virus (EBV) have continued in two major areas. In collaboration with investigators of the NEI we are carrying out a longitudinal study of the frequency of EBV-infected B cells in the peripheral blood of patients receiving long-term treatment with cyclosporin A (CSA). Previously, we have shown that CSA blocks all immunoregulatory T-cell activity for EBV in vitro, and since EBV-induced B-cell lymphomas have occurred in patients on CSA therapy, such clinical studies might prove useful in understanding malignant conversion in this setting. Our longitudinal studies have revealed that the frequency of B lymphocytes carrying EBV increases progressively with the duration of CSA treatment and has reached an alarming 1,000-fold increase in one patient. Detailed evaluation and analysis of these clinical studies may help regulate immunosuppressive therapy within a safe range before overt malignancy develops. Our studies of patients with chronic mononucleosis are coming to an end with the conclusion of a double-blind trial of acyclovir therapy. Unfortunately, on the expanded population of patients seen for this therapy trial we have been unable to reproduce our initial reported finding of a defect in suppressor cell activity in these patients.

We have continued to study the immunosuppressive effects of the organic acid, succinylacetone (SA). The compound appears to substantially inhibit primary antibody responses to SRBC in rats and is effective in presenting graft-versus-host disease (GVHD). Preliminary experiments also indicate that it may reverse established GVHD if given early, after symptoms appear. Importantly, SA can be used as the sole immunosuppressive drug in a model of total allogeneic bone marrow transplantation. In these experiments, full engraftment and survival in the absence of GVHD was seen in 11 of 12 animals tested, demonstrating that SA may have chemical utility in transplantation.

The major thrust of the work of the Cellular Immunology Section has been directed toward the development of techniques in reagents for the use of gene therapy in the treatment of human disease. The most likely initial candidate disease in which gene therapy will be employed is the form of severe combined immune deficiency (SCID) caused by deficiency of the purine salvage enzyme, adenosine deaminase (ADA). Our lab has established unique T-lymphocyte cell lines from four patients with ADA deficiency using the T-lymphotropic-transforming virus HTLV-I. Since the primary site of pathology in ADA deficiency is the T cell, these lines have provided us with an exceptionally valuable resource for learning more about the fundamental biochemical defect of this disease as well as giving us an in vitro test system for evaluating the efficiency of gene transfer and for the expression of the transferred genes leading to functional reconstitution of the cells. Although no T cells were detected by cell surface markers or functional assays in these ADA-deficient patients, HTLV-I transformation of bone marrow or peripheral blood mononuclear cells resulted in the growth of T-cell lines from each patient studied. The cells bore mature T-cell markers and clones of these cells, and each had distinctive T-cell receptor beta change gene rearrangements. The level of ADA in each line was less than 1% of normal. Furthermore, the growth of each line was inhibited by deoxyadenosine at a concentration 100-fold less than that needed to inhibit the growth of normal T-cell lines.

In collaboration with Dr. French Anderson of the NHLBI and Eli Gilboa at Princeton University, we have been using these ADA-deficient T-cell lines to study retroviral-mediated transfer of the human ADA gene. A murine retrovirus has been modified by the removal of its GAG, POL, and ENV coding sequences and the insertion of a bacterial neomycin resistance gene and the human ADA cDNA promoted from an SV40 early region promoter. The ADA-deficiency T cells were infected with this ADA vector (called SAX) under various experimental protocols and then analyzed for ADA protein levels and deoxyadenosine resistance. Our average level of gene transfer with this vector is 30% as measured by DNA dot blotting of individual T-cell clones derived immediately following treatment of the ADA-deficiency T-cell lines with the SAX vector. ADA levels in the vector DNA-positive cells become normal as does the resistance to the toxic effects of dyoxyenysyne. Over 90% of the clones containing at least a single copy of the vector produced functional human ADA at normal levels. Therefore, this gene delivery system is very effective in transferring genes to human T lymphocytes and the transferred genes are expressed very efficiently, holding promise that gene therapy for ADA deficiency may be possible within the not too distant future.

Toward this goal, we have begun collaborative studies with Dr. Richard O'Reilly at the Memorial Sloan-Kettering Cancer Center in New York and Dr. Arthur Neihius at the NHLBI using a bone marrow transplantation protocol in monkeys to test our gene transfer system. These studies, although still preliminary, have demonstrated that we can successfully induce the production of some human ADA in SAX vector monkeys, again demonstrating the potential for this mode of gene transfer.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00416-13 MET

PERIOD COVERED

October 1, 1985 through September 30, 1986

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
	Marie Gelato	Medical Staff Fellow	MET, NCI
	Wieland Kiess	Guest Researcher	MET, NCI
	Matthew M. Rechler	Senior Investigator	NIADDK
	Wayne Anderson	Senior Investigator	LTIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

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

☐ (a2) Interviews

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

In order to sort out the respective roles of the type I and type II insulin-like growth factor (IGF) receptors in mediating biologic responses, we developed a blocking antibody to the type II receptor. We immunized rabbits with type II receptor purified to homogeneity from rat chrondosarcoma chondrocytes. The resulting antiserum blocks the binding of radiolabeled IGF-II to the type II receptor of a variety of cells and membrane preparations. The antiserum does not block the binding of insulin to the insulin receptor nor does it block the binding of IGF-I to the type I receptor. Based on correlations of dose response curves for IGF-II binding and IGF-II-stimulated biologic responses, others had proposed that the type II receptor was important in mediating IGF-II-stimulated growth responses in L6 myoblasts. Accordingly, we examined the ability of IgG prepared from the antireceptor antiserum to mimic or block IGF-II-stimulated biologic responses in L6 myoblasts. We find that concentrations of anti-type II receptor IgG that are sufficient to completely block IGF-II binding do not mimic or block IGF-II-stimulated glucose or amino acid uptake or thymidine incorporation into DNA. The antireceptor IgG does block the degradation of IGF-II in the medium, presumably by blocking receptor-mediated internalization. In the circulation, IGFs are associated with "binding" or "carrier" proteins. There are two size classes of carrier proteins, a M_r=40,000 protein which is the only carrier protein in fetal blood and a M_r=150,000 protein which is under growth hormone control and is found in postnatal blood. There are conflicting data regarding the structure of the 150,000 binding protein. To resolve these conflicting models, we have chemically crosslinked radiolabeled IGF-I and IGF-II to preparations of the 150,000 binding protein and analyzed the radioligand-binding protein complexes by sodium dodecyl sulfate gel electrophoresis and autoradiography. Our data suggest that the 48,000 species generated from the 150,000 binding protein is not simply a dimer of a 25,000 species, contrary to the prediction of the six subunit model of the 150,000 binding protein.

GPO 914-918

Project Description

Major Findings:

1. Type II IGF Receptor Purification

We have improved the recovery of type II receptor from Triton X100 solubilized rat placental membranes by constructing an affinity column with a rat IGF-II species with higher affinity for the type II receptor (rat IGF-II, M_r=7450 rather than rat IGF-II, M_r=8700). We have been able to purify several hundred micrograms of type II receptor from approximately 100 rat placentae with a recovery approaching 50%. The purified receptor is being used to obtain partial amino acid sequence data on cleaved fragments in order to enable us to construct nucleic acid probes for use in cloning the cDNA for the type II receptor.

2. Development of a Polyclonal Blocking Antibody for the Type II Receptor

We immunized rabbits with highly purified type II receptor and obtained an antiserum directed against the type II receptor. The antiserum blocks the binding of radiolabeled IGF-II to the type II receptor. Interestingly, if the receptor binding site is occupied by IGF-II, the antiserum does not interact with the receptor as determined by immunoprecipitation with second antibody, suggesting that the antibodies in the polyclonal antiserum are only directed against the ligand binding site. Experiments in which crude preparations of type II receptor (Triton X100 solubilized membranes) were electrophoresed on gels, transferred to nitrocellulose, and the nitrocellulose exposed in turn to antibody and ^{125}I-Protein A, demonstrated that the antiserum was only directed against the type II receptor. The antiserum did not block the binding of ^{125}I-insulin to the insulin receptor nor block the binding of ^{125}I-IGF-I to the type I IGF receptor. We have utilized an IgG fraction purified from this antiserum by Protein A affinity chromatography to attempt to define the functional role of the type II receptor. Based on correlation of dose-response curves for binding versus biologic response (glucose and amino acid uptake) in a rat myoblast cell line (L6), others had proposed that the type II receptor was involved in mediating IGF-II-stimulated glucose and amino acid uptake. We found, however, that anti-type II receptor IgG did not behave as an agonist or an antagonist for IGF-II-stimulated glucose and amino acid uptake in the L6 myoblasts. Presumably, the type I receptor mediates IGF-stimulated biologic responses in these cells. We did find that the anti-receptor IgG blocked the degradation of IGF-II in the medium, presumably by inhibiting receptor-mediated internalization.

3. Structure of the 150,000 IGF Binding Protein

In the circulation, IGFs are associated with "binding" or "carrier" proteins. There are two size classes of carrier proteins, a M_r=40,000 protein which is the only carrier protein in fetal blood and a M_r=150,000 protein which is under growth hormone control and is found in postnatal blood. There are conflicting data regarding the structure of the 150,000 binding protein. Some data suggest that the 150,000 species is simply made up to six 25,000 binding subunits, whereas other data suggest that the 150,000 carrier protein is made up of a 40,000-60,000 binding subunit and a larger nonbinding subunit. To resolve these conflicting models, we have chemically crosslinked radiolabeled IGF-I and IGF-II to prepara-

tions of the 150,000 binding protein and analyzed the radioligand-binding protein complexes by sodium dodecyl sulfate gel electrophoresis and autoradiography. Our data suggest that the 48,000 species generated from the 150,000 binding protein is not simply a dimer of a 25,000 species, contrary to the prediction of the six subunit model of the 150,000 binding protein. We are attempting to raise monoclonal antibodies to the various binding subunits generated from the 150,000 species to further resolve the various models for the 150,000 binding protein structure.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04017-08 MET

PERIOD COVERED

October 1, 1985 through September 30, 1986

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	Senior Investigator	MET, NCI
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Bernard Boutin	Guest Researcher	MET, NCI
David K. Wagner	Medical Staff Fellow	MET, NCI
Luisa Marcon	Guest Researcher	MET, NCI
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COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

Immunophysiology

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	5	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.)*

Studies were undertaken to investigate the maturation and immunoregulation of T-cell and B-cell responses in normal individuals and patients with immune deficiency diseases. A hybridoma-derived monoclonal antibody, termed 7G7/B6, was produced and characterized which binds to the interleukin-2 receptor (IL-2R) on human lymphocytes at a site distinct from that which binds to IL-2 and to the monoclonal anti-IL-2R antibody, anti-Tac. Using these two antibodies, a "sandwich" enzyme-linked immunosorbent assay (ELISA) has been developed to quantitatively measure soluble IL-2R. Using this ELISA, we have found IL-2R in the sera of all normal individuals tested and abnormally elevated levels of IL-2R in the sera of patients with human T-lymphotropic retrovirus (HTLV)-related disorders, including the adult T-cell leukemia (ATL) and hairy cell leukemia (HCL). In ATL and HCL, a lowering of the serum level of IL-2R was associated with favorable clinical responses to monoclonal antibody therapy and conventional chemotherapy in ATL and recombinant alpha-interferon therapy in HCL. Serum IL-2R levels were high in patients with HTLV-III-related syndromes--AIDS, ARC, and LAS. Disease activity in patients with autoimmune syndromes such as rheumatoid arthritis and lupus erythematosus was paralleled closely by changes in serum IL-2R. IL-2R were also found in the urine of all normal individuals tested. Like the serum IL-2R, urinary IL-2R was capable of binding IL-2 and had an apparent molecular weight of 40-45 kDa, slightly less than cell-associated IL-2R. Soluble IL-2R may play an immunoregulatory role *in vivo*, and the levels of receptor may be indicative of alterations in immune reactivity.

GPO 904-917

Project Description

Major Findings:

Employing somatic cell hybridization techniques, a murine hybridoma-derived monoclonal antibody (7G7/B6) was produced which specifically binds to the human interleukin-2 (IL-2) receptor (IL-2R) at a site distinct from that binding IL-2 or other monoclonal antibodies binding to this receptor, such as the antibody termed anti-Tac. Using 7G7/B6 and anti-Tac, a "sandwich" enzyme-linked immunosorbent assay (ELISA) was developed to measure IL-2R in solution. Using this assay, we were able to demonstrate that activated normal peripheral blood mononuclear cells release a soluble form of the IL-2R into tissue culture supernatants during in vitro culture. Furthermore, we were able to document that certain tumor cell lines constitutively released IL-2R into their tissue culture supernatants. This was particularly true of human T-lymphotropic retrovirus (HTLV)-I-infected T-cell lines. The HTLV-I dependence of this observation was documented by infecting a normal T-cell line with HTLV-I and observing 10-fold rises in the amount of soluble IL-2R generated. In addition to tumor cell lines of T-cell origin, Epstein-Barr-transformed B-cell lines also released soluble IL-2R. An analysis of the T-cell subsets from normal peripheral blood responsible for the release of soluble IL-2R in vitro demonstrated that CD4-positive T cells and CD4-negative T cells released roughly equal amounts of IL-2R and that normal activated B cells also released IL-2R. Studies of the maturation of this response revealed that neonatal cord blood T cells were fully capable of expressing both cell-associated and soluble IL-2R following cellular activation in vitro. The molecular weight of the soluble IL-2R was found to be 40-45 kDa, roughly 10 kDa smaller than the mature cell surface form of the receptor. In addition, the soluble form of IL-2R was shown to bind to IL-2R very efficiently and to inhibit IL-2-dependent immune responses.

The discovery of this soluble form of the IL-2R in vitro led to studies of soluble IL-2R in several body fluids in various disease states. Soluble IL-2R were measurable in the serum of all normal individuals tested (mean 186 U/ml, range 83-335). Other studies on the level of IL-2R in cord blood serum revealed values comparable to that in adult serum--suggesting that there may not be major age-related differences in levels of serum IL-2R. In accord with the in vitro data relative to HTLV-I-infected T cells, elevated levels (mean 35,300, range 9,500-69,500) of soluble IL-2R were found in the sera of all patients with the adult T-cell leukemia (ATL), a leukemia associated with HTLV-I infection in man. Levels of serum IL-2R comparable to that seen in patients with ATL were also found in the sera of patients with a different leukemia of B cells known as hairy cell leukemia (HCL), which has been associated with infection by HTLV-II, a virus related to HTLV-I. Serum levels of more than 2000 U/ml were found in other patients with leukemias/lymphomas, including 3 of 7 patients with Hodgkin's disease, 12 of 21 patients with the Sézary syndrome (an HTLV-I-negative proliferation of mature T cells), and 6 of 20 patients with chronic lymphocytic leukemia. In patients with ATL and HCL, favorable clinical responses to therapy were associated with reductions in serum IL-2R levels. This included responses to anti-Tac and conventional chemotherapy in ATL patients and recombinant alpha-interferon in HCL patients. These results suggest that serum levels of IL-2R will be useful in diagnosing various malignancies, monitoring patients for response to therapy, and surveillance for early relapse following therapy. We have also found that treatment of

cancer patients with recombinant DNA-derived IL-2 resulted in a 100- to 1000-fold rise in the levels of serum IL-2R, suggesting that the measurement of serum IL-2R might be a means to monitor therapy with other agents capable of modifying immunologic responsiveness (biological response modifiers). We also examined the serum levels of IL-2R in patients with several syndromes related to LAV/HTLV-III infection. Relative to heterosexual and healthy homosexual controls, significantly elevated levels ($p < 0.01$) of soluble IL-2R were found in the sera of patients with the acquired immune deficiency syndrome (AIDS), those with the lymphadenopathy syndrome (LAS), and the AIDS-related complex (ARC). Thus, elevated levels of serum IL-2R might be an early indicator of LAV/HTLV-III infection and by binding to IL-2 might contribute to the infections observed in AIDS patients by inhibiting immune responses to HTLV-III itself as well as to other foreign antigens. Certain other poorly defined inflammatory diseases, such as sarcoidosis, rheumatoid arthritis, and systemic lupus erythematosus, were associated with elevated levels of serum IL-2R, and the levels of receptor paralleled disease activity.

Soluble IL-2R were also found in the urine of all normal individuals tested (n=8), and the mean value in urine (118 U/ml) was not significantly different ($p > 0.05$) than the serum levels (178 U/ml) in these same individuals. The urinary IL-2R, like the serum IL-2R, has an apparent molecular weight of 40-45 kDa by SDS-PAGE analysis and is fully capable of binding to IL-2. Patients with ATL who had elevated serum levels of IL-2R also had significantly elevated urinary levels of IL-2R. The finding of IL-2R in normal urine suggests that monitoring urinary IL-2R levels might be of value in monitoring immune-mediated kidney disorders.

An analysis of lymphocyte growth factor receptors in serum and other body fluids may be a sensitive means to monitor individuals with disorders of immune maturation and regulation.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04018-10 MET

PERIOD COVERED

October 1, 1985 through September 30, 1986

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)*

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Basil Golding	Medical Staff Fellow	FDA

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

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

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.)*

Our laboratory has concentrated on the purification and characterization of a unique 85 kilodalton immunosuppressive glycoprotein termed uromodulin which was originally isolated from human pregnancy urine. This material blocks antigen specific proliferative assays which are dependent upon T cell proliferation at concentrations of 10-10M. Uromodulin is also a specific inhibitor of interleukin 1 and blocks both thymocyte and IL-1 dependent T cell lines at concentrations of 10-10M. Interestingly Uromodulin is a specific and high affinity ligand for both recombinant IL-1 alpha and IL-1 beta, and it appears that this is the mechanism by which uromodulin is able to regulate the activity of IL-1. We have expanded these studies and recently have been able to sequence over 200 amino acids of native uromodulin. Using this sequence data we have recently isolated what we believe is a full length message for uromodulin. This message has been inserted into several different vectors and is in the process of being expressed in several transient expression systems. Preliminary data suggest that a subline of COS-7 is able to synthesize uromodulin. We have also been characterizing the mechanism of binding of IL-1 to uromodulin and find that IL-1 actually recognizes carbohydrate sequences expressed by uromodulin. A number of studies have been utilized including digestion with endoglucoaminidase F, pronase, and isolation of released fragments by HPLC and high performance thin layer chromatography to further characterize the carbohydrate sequence responsible for binding to IL-1. Clinical studies have also been instituted. We have developed a number of ELISA assays based on several monoclonal antibodies generated in our laboratory and preliminary evidence suggests that uromodulin in elevated in a number of clinical conditions which are associated with elevations of IL-1.

GPO 914-918

Our laboratory has concentrated on the purfication and characterization of a unique 85 kilodalton immunosuppressive glycoprotein termed uromodulin which was originally isolated from human pregnancy urine. This material blocks antigen specific proliferative assays which are dependent upon T cell proliferation at concentrations of 10-10M. Uromodulin is also a specific inhibitor of interleukin 1 and blocks both thymocyte and IL-1 dependent T cell lines at concentrations of 10-10M. Interestingly Uromodulin is a specific and high affinity ligand for both recombinant IL-1 alpha and IL-1 beta, and it appears that this is the mechanism by which uromodulin is able to regulate the activity of IL-1. We have expanded these studies and recently have been able to sequence over 200 amino acids of native uromodulin. Using this sequence data we have recently isolated what we believe is a full length message for uromodulin. This message has been inserted into several different vectors and is in the process of being expressed in several transient expression systems. Preliminary data suggest that a subline of COS-7 is able to synthesize uromodulin. We have also been characterizing the mechanism of binding of IL-1 to uromodulin and find that IL-1 actually recognizes carbohydrate sequences expressed by uromodulin. A number of studies have been utilized including digestion with endoglucoaminidase F, pronase, and isolation of released fragments by HPLC and high performance thin layer chromatography to further characterize the carbohydrate sequence responsible for binding to IL-1. Clinical studies have also been instituted. We have developed a number of ELISA assays based on several monoclonal antibodies generated in our laboratory and preliminary evidence suggests that uromodulin is elevated in a number of clinical conditions which are associated with elevations of IL-1.

SIGNIFICANCE OF THESE FINDINGS:
These observations have a number of very significant implications not only from a theoretical and scientific aspect but also from a clinical and practical aspect. First our data demonstrate that interleukin-1 is a lectin. This is to our knowledge the first example of a human lectin purified to homogeneity and available as a recombinant protein. This observation helps to explain the protean activities of interleukin 1. Second uromodulin is to our knowledge the only know specific inhibitor of IL-1 which is available as a homogeneous purified glycoprotein (we predict that within the next few months it too will be available as a recombinant protein since our evidence suggests that a full length functional message is now available). A very large effort is under way in the pharmaceutical industry and clinical laboratories to isolate and characterize inhibitors of IL-1. Uromodulin will be an invaluable tool to further disect the activities of interleukin 1; furthermore we believe that uromodulin may have clinical usefulness as an inhibitor of IL-1. Our own data demonstrate that uromodulin appears to be non-toxic and preliminary studies demonstrate that it is able to protect animals from lethal challenge with endotoxin. Finally, our data suggest that carbohydrate fragments of uromodulin are also capable of mediating biologic activity and this opens up an entire area of research into potential clinically useful immunoregulatory oligosaccharides derived from this most interesting compound.

Publications:

Muchmore, A.C., Decker, J.M.: Uromodulin: A Unique 85-kilodation Immunosuppressive Glycoprotein Isolated from Urine of Pregnant Women: Science, 1985, pp. 479-481.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04020-09 MET

PERIOD COVERED

October 1, 1985 through September 30, 1986

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

Antigen-Specific T-Cell Activation and Genetic Control of Immune Responses

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

PI:	Jay A. Berzofsky, M.D., Ph.D.	Senior Investigator	MET, NCI
	Kemp B. Cease, M.D.	Medical Staff Fellow	MET, NCI
	Shoichi Ozaki, M.D., Ph.D.	Visiting Fellow	MET, NCI
	Masaharu Kojima, Ph.D.	Visiting Fellow	MET, NCI
	Sara Brett, Ph.D.	Guest Worker	MET, NCI
	Michael Good, M.D., Ph.D.	Guest Worker	MET, NCI; LPD, NIAID
	James Cornette, Ph.D.	Prof. of Mathematics	Iowa State U.
	Hanah Margalit, Ph.D.	Visiting Fellow	LMB, NCI

COOPERATING UNITS *(if any)*

See Continuation Sheet attached.

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

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INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10.1	8.1	2

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☐ (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.)*

We have studied the mechanism of T-cell activation by antigen, and the MHC-linked genetic control thereof. In contrast to antibodies, the T-cell response is dominated by a few immunodominant antigenic sites, which represent regions on which a polyclonal T-cell response is focused. We found that the immunodominance of a site can be determined both by extrinsic factors, such as the major histocompatibility (MHC) antigens of the responder, and by intrinsic factors in the antigen structure. The two immunodominant sites of myoglobin we found around residues 109 and 140 were recognized with different MHC antigens, I-A^d and I-E^d, respectively. With synthetic peptides, we narrowed these sites to 106-118 and 133-146, respectively. Both of these are amphipathic alpha helices. We examined the 23 known immunodominant sites from 12 different proteins, and found that 18 of these have a periodicity of hydrophobicity that would make them amphipathic if they fold as an alpha helix. We have developed an algorithm to search for such sequences and are using this to predict T-cell sites from AIDS and malaria proteins, for purposes of developing synthetic vaccines. Corresponding peptides are being prepared and tested. Using a biotinylated immunodominant peptide, we showed that the peptide was on the surface of the presenting cell, accessible to macromolecules such as avidin. We found that the cloned, antigen-specific, Ia-restricted, $L3T4^+$ T cells also killed tumor but not normal presenting cells. The induction was antigen-specific, but the effector phase was not, although it was preferentially inhibited by specific cold targets. This may represent a novel type of tumor surveillance. We also found that on antigen stimulation, the T-cell clones release soluble IL-2 receptor, secrete a new lymphokine that stimulates IL-1 secretion by macrophages, and increase their expression of Fc receptors for IgD (also induced by IgD itself). We also found a novel population of $L3T4^+$, IL-2 receptor-bearing T cells in unstimulated normal spleen cells, whose level is controlled by several genes including one mapped to chromosome 7.

GPO 914-918

ntinuation Sheet for PHS 6040

OPERATING UNITS:

Charles DeLisi, Ph.D.	Director, Health and Environmental Research	DOE
Ira Berkower, M.D., Ph.D.	Senior Investigator	OB, FDA
Gene M. Shearer, Ph.D.	Senior Investigator	IB, NCI
Louis H. Miller, M.D.	Chief, Malaria Section	LPD, NIAID
Scott Durum, Ph.D.	Senior Investigator	BRMP, FCRF, NCI
Richard Coico, Ph.D.	Asst. Prof., Dept. Pathol.	NYU
Jeanette Thorbecke, Ph.D.	Professor, Dept. Pathol.	NYU
Susan O. Sharrow	Investigator	IB, NCI

Project Description

A primary goal of the laboratory has been to understand and ultimately manipulate the immune response at an antigen-specific level. To do so, we have been studying the mechanisms of immune response (Ir) gene function and T-cell activation by antigen in association with major histocompatibility (MHC) antigens. In contrast to antibodies, the cellular immune response, as we have found, is dominated by T cells specific for a few immunodominant antigenic sites. The immunodominance is due to a focusing of a polyclonal response onto a single region of the molecule, rather than to the preemption of the response by a single dominant clone. We have shown that immunodominance of a site depends both on factors intrinsic to the antigen, such as helical amphipathicity (the property of having one side hydrophobic and the other hydrophilic--see below), and on factors extrinsic to the antigen, especially the MHC antigens of the responding individual, and thus its Ir genes. In collaboration with Dr. Charles DeLisi and his group, we have found that the majority of known immunodominant sites recognized by helper T cells have a common property, namely that if they fold into a helix, that helix would be amphipathic. They have developed a computer algorithm to predict such sites from the protein sequence alone, which correctly identifies 18 of 23 known immunodominant T-cell sites on 12 proteins. The refinement of this algorithm required development of new mathematical approaches and the comparison of 38 hydrophobicity scales and optimization by a new eigenvector approach. The ability to predict T-cell antigenic sites may be useful for development of synthetic vaccines, and therefore we have applied this approach to the prediction of sites on the AIDS virus and malaria parasite. We have synthesized several AIDS virus peptides and are testing these in collaboration with Dr. Gene Shearer. In addition, we are testing predicted sites on the malaria circumsporozoite protein in collaboration with Dr. Louis Miller's group and have shown Ir gene control of the response to these peptides. As T-cell recognition is critical not only for cellular immune defense but also for T-cell help for a memory antibody response, the presence of a site recognized by T cells should be essential for a successful vaccine to any protein antigen. This is the first algorithm to be able to predict such sites.

In addition to this practical application, the finding that T cells tend to recognize amphipathic structures suggests some fundamental chemical rules in T-cell recognition. It seems likely, from our studies, that the T-cell receptor interacts with the hydrophilic part of the structure, while the hydrophobic part interacts with some structure on the presenting cell surface, such as an MHC antigen or the membrane itself. The peptide antigen would be expected to be accessible on the surface of the presenting cell, but attempts in several laboratories to provide this by blocking T-cell responses with antibody to antigen have given equivocal results. We have gotten around this problem by attaching biotin specifically to the N-terminal amino group of an immunodominant antigenic peptide, and showing that avidin, which binds biotin with enormously high affinity, can block antigen presentation. The blocking occurs even after 8 hours of pre-exposure of the presenting cell to antigen, washing, and a further 16 hours of culture to allow antigen to reach the surface. It also occurs in the presence of an inhibitor of intracellular trafficking. Thus, the immunologically relevant form of the peptide is present on the cell surface in a form accessible to macromolecules the size of avidin (55 x 55 x 41 Å).

In the course of these studies, we found that antigen-specific cloned helper T cells could also kill tumor antigen-presenting cells (APC). The activation requires antigen processing and is MHC-restricted. The effector mechanism is nonspecific, but requires cell contact (or proximity) with the T cell and secretion or injection of a short-acting lymphokine that is synthesized de novo only during antigen-specific stimulation. Thus, the killing acts paradoxically on bystander targets but displays specific cold-target blocking. This may be a new function of "helper" T cells as effector cells in tumor surveillance, as normal cells have little or no susceptibility to this form of killing.

Also, in the course of these studies, in collaboration with the group of Dr. Scott Durum, we discovered that helper T-cell clones, on stimulation with antigen and presenting cells, induce interleukin-1 (IL-1) secretion by macrophages. This induction is mediated by a new low-molecular weight lymphokine secreted by the T-cell clones, which is now being characterized. We have also demonstrated a new population of IL-2 receptor-bearing T cells in unstimulated spleen cells, exclusively in the $L3T4^+$ $Lyt2^-$ subpopulation, and genetically regulated by non-MHC genes, especially one mapped to chromosome 7 of the mouse. Also, in collaboration with Dr. David Nelson's group, we showed that on activation by antigen, T-cell clones release a soluble form of the interleukin-2 (IL-2) receptor. It is hoped that these studies of T-cell activation and new T-cell effector functions will provide a basis for understanding T-cell tumor surveillance and for development of new approaches to immunotherapy aimed at T-cell immunity.

Summaries of Specific Projects:

1. Analysis of an Immunodominant T-Cell Antigenic Site with Synthetic Peptides: Focusing of a Polyclonal Response.

The T-cell response to sperm whale myoglobin in the H-2^d haplotype has been shown to be largely focused on a limited region around glutamic acid 109 recognized in association with I-A^d. T-cell clones 9.27 and 1.2, derived from B10.D2 and (B10.D2 x B10.BR)F_1 mice, respectively, have been previously shown to reflect this specificity and MHC restriction. In this study we have used a panel of synthetic peptides from the region 102-118 of sperm whale myoglobin to characterize the specificities of these representative clones. The segment from 106-118 was found to represent a consensus region for recognition by both clones. However, significant differences between clones in the hierarchy of responsiveness to peptides within the panel were observed. In as much as the peptide and the I-A^d molecule remain constant, these differences derive from differences in how each T-cell receptor interacts with the antigen. Thus, they place specific constraints on structural models of antigen as it exists as part of the stimulation complex for the T-cell receptor. This peptide segment is an amphipathic alpha helix in native myoglobin, meaning that one side is hydrophobic and the other hydrophilic. It is one of the prototype cases that led us to find that amphipathic helices constitute the majority of immunodominant sites recognized by helper T cells. It is likely that the peptide will refold into an amphipathic helix stabilized by the interface at the surface of the presenting cell. When such secondary conformation is considered, these data are consistent with a model of multiple T-cell specificities arising from multiple "views" of a single antigen conformation at a single Ia binding site and do not require postulation of multiple conformations or binding sites.

Additionally, the finding of distinct specificities suggests that the immunodominance of this site depends not on the dominance of a single clone, but on the focusing of polyclonal response on a single region of the molecule in association with $I\text{-}A^d$. The reason for immunodominance of this particular region of the protein may thus depend on specific intrinsic features of the site such as potential to form an amphipathic alpha helix as well as extrinsic factors such as binding properties of the I-A molecule.

2. The Analysis and Optimization of Hydrophobicity Scales for Detection of Amphipathic Alpha Helices (with Charles DeLisi's group).

Protein segments that form amphipathic alpha-helices in their native state have periodic variation in the hydrophobicity values of the residues along the segment, with a 3.6 residue per cycle period characteristic of the alpha-helix. The assignment of hydrophobicity values to amino acids (hydrophobicity scale) affects the display of periodicity. Thirty-eight published hydrophobicity scales were compared for their ability to identify the characteristic period of alpha-helices, and an optimum scale for this purpose was computed using a novel eigenvector approach.

We compared the usual method for detecting periodicity based on the discrete Fourier transform with a method based on a least squares fit of an harmonic sequence to a sequence of hydrophobicity values. The two become equivalent for very long sequences, but for shorter sequences with lengths commonly found in alpha-helices, the least squares procedure gives a more reliable estimate of the period. The analogue to the usual Fourier transform power spectrum is the "least squares power spectrum"--the sum of squares accounted for in fitting a sinusoid of given frequency to a sequence of hydrophobicity values. Both spectra were used to analyze a crystallographic data base of alpha and beta structures.

The sum of the spectra of the alpha-helices in our data base peaks at 97.5°, and approximately 50% of the helices can account for this peak. Thus, approximately 50% of the alpha helices appear to be amphipathic, and of those that are, the dominant frequency at 97.5° rather than 100° indicates that the helix is slightly more open than usually thought, with the number of residues per turn closer to 3.7 than 3.6.

The alpha amphipathic index, the key quantity in our analysis, measures the fraction of the total spectral area that is under the 97.5° peak, and is a consistent characteristic of hydrophobicity scales for different sets of helices. Our optimized scale maximizes the amphipathic index and has a correlation of 0.85 or higher with nine previously published scales. The most surprising feature of the optimized scale is that arginine tends to be hydrophobic; i.e., in the crystallographic data base it has a tendency to be on the hydrophobic face of the amphipathic helix. Although the scale is optimal only for predicting alpha-amphipathicity, it also ranks high in identifying beta-amphipathicity and in distinguishing interior from exterior residues in a protein.

We factored the expressions for the power spectra so that the helical sequence information is isolated from the hydrophobicity scale. The factor containing only helical sequence information also identifies the 97.5° frequency, thus confirming a 3.7 residue per turn spacing independently of hydrophobicity scales.

3. Development of an Algorithm to Predict Immunodominant T-Cell Epitopes (with Charles DeLisi's group).

The computational scheme of DeLisi and Berzofsky (1985) used to associate alpha-helical amphipathicity with known immunodominant helper T-cell antigenic sites in proteins has been modified to produce algorithms for predicting T-cell antigenic sites. A predictive algorithm identifies segments of proteins that, if isolated as polypeptides, could form amphipathic alpha-helices and thus have a high probability of being an immunodominant T-cell site in a protein.

The input data to an algorithm is the primary amino acid sequence of a protein. Each amino acid is assigned a hydrophobicity value (according to some scale) and the resulting sequence of numbers is searched for segments within which the numbers vary with a period of approximately 3.6 residues per cycle. The performance of an algorithm depends on the hydrophobicity scale selected and the mathematical scheme used to detect 3.6 residue per cycle periodicity. To detect periodicity, we used the discrete Fourier transform and a method of least squares fit of a sinusoid to a sequence of hydrophobicity values.

The algorithms were tested on a data base of 23 known T-cell antigenic sites in 12 proteins. The predicted amphipathic segments were compared with the locations of the known T-cell sites, the number of "matches" recorded, and the probability (p) of getting that number of matches or more by chance alone computed. A good algorithm has a large number of matches with a low value of p. The best algorithms so far developed use the Fauchere-Pliska hydrophobicity scale and the method of least squares, and they correctly predict 17 or 18 of the 23 known T-cell sites with a probability of doing so by chance alone ranging from .0006 to .0025.

4. Demonstration that Immunologically Relevant Antigen is Present on Presenting Cells in a Form Accessible to Macromolecules.

Studies of the association of antigen with APC have been complicated by antigen processing requirements, but recent studies have suggested that immunologically relevant antigen should be present on the APC surface. Nevertheless, blocking of antigen presentation with antibody to the antigen has not been demonstrable in most systems. To study this problem we developed a system using avidin to block presentation of N-terminal biotinylated synthetic peptide 132-146 of sperm whale myoglobin (B132) to a murine T-cell clone specific for this site in association with I-E^d. Greater than 95% specific inhibition was observed with doses of B132 equipotent to unmodified peptide. Specific blocking could be observed: (a) after pulsing APCs with antigen, washing, and incubating for 16 hours prior to addition of avidin and T cells to assure adequate time for intracellular trafficking and maximal display of antigen on the cell surface or (b) when monensin is present during the antigen pulse to inhibit such traffic. Therefore, the inhibition is occurring at the cell surface unless dissociation and reassociation are constantly occurring. To distinguish these, B10.GD APCs (I-E^d negative) were pulsed with antigen and cocultured with B10.D2 APCs (I-E^d positive). No detectable antigen presentation resulted. Thus, minimal dissociation and reassociation between antigen and APC occurs and, consequently, blocking by extracellular solution phase binding of avidin to antigen is unlikely. Taken together, these data suggest that the blocking is occurring at the cell surface. Thus, under physiologic conditions, immunologically relevant antigen necessary for T-cell activation appears

to be present on the APC surface and is freely accessible to macromolecules the size of avidin. These findings hold specific structural implications for models of antigen presentation for T-cell recognition.

5. Killing by Helper T Cells: Activation Requirements and Mechanism of Killing.

Killing by helper T cells is a novel and unexpected function only recently observed in several laboratories, including our own. The mechanism of helper T-cell-mediated killing was intensively investigated. Myoglobin-specific, Ia^d-restricted cloned helper T cells and T hybridomas directly killed Ia^d-bearing, myoglobin-pulsed B lymphoma targets and killed bystander targets only in the presence of specific APC. The induction of the killing required recognition of processed antigen in the context of class II MHC molecules on the surface of APC. Despite the specific induction of the killing, the bystander killing suggests a nonspecific lytic mechanism. Consistent with this interpretation, the direct killing could be inhibited only by cold-specific targets in a dose-dependent manner, whereas the bystander killing could be blocked by both specific and nonspecific targets. The cold target inhibition seems to be due to interference with effector-to-target contact or proximity rather than due to high-dose suppression of T-cell activation. The helper T cells appear to kill targets by synthesizing short-range soluble factor(s) with nonspecific killing activity de novo during the effector phase, but only while signal transduction is occurring through the interaction of the T-cell receptor with specific ligands, because (1) no long-lived killing activity was detected in the T-cell supernatant, (2) ongoing stimulation of T cells by specific stimulators was required for the bystander killing to occur even after preactivation of the T cells, and (3) the killing could be inhibited by adding cyclosporin A or cycloheximide during the 6-hour effector phase.

Among the various APC, there existed a dissociation between the capacity to stimulate T cells and the capability to be killed and to inhibit specific killing. Only Ia-bearing B lymphoma cells and macrophage tumor cells could inhibit the specific killing. The most efficient cold target inhibition was mediated by cells which (1) carried the correct combination of antigen and Ia to bind with high affinity to the helper T cells and (2) bore receptors for the putative cytotoxic lymphokine, in that they were susceptible to killing. Therefore, the mechanism of cold target inhibition appears to be absorption or consumption of a short-acting cytotoxic lymphokine by cells which must be able to interact closely with the effector cell. Normal spleen B cells, however, could not inhibit specific killing even after lipopolysaccharide (LPS) stimulation, despite their capability for activating the helper T cells. The dissociation is in keeping with the fact that, in our hands, the normal B cells could not be killed by the helper T cells even after stimulation by LPS. Thus, although killing by helper T cells may play a negative feedback role in the normal immune response, our data raise the possibility that the helper T-cell-mediated killing may contribute to the immune surveillance against malignancy by virtue of the preferential killing of tumor cells either directly or indirectly.

6. Antigen-specific Induction of a Soluble Lymphokine from Helper T-Cell Clones that Induces Macrophage IL-1 Secretion (with Scott Durum's laboratory).

T-cell activation is widely believed to depend on IL-1 provided by APC. Since IL-1 is not a constitutive product of APC, we examined the features of its produc-

tion during the interaction of murine T-cell clones and APC. We observed that IL-1 was detectable in supernatants of most myoglobin-specific T-cell clones grown with APC and antigen. Two of these T-cell clones displayed exceptionally high levels of IL-1 in their supernatants and these same clones demonstrated the unusual restriction to I-E^k, which is a low responder type for sperm whale myoglobin. One of these clones was characterized further as to mechanism. This clone rapidly induced IL-1 production in the APC population (detectable at 4 hours of coculture). IL-1 induction was antigen dependent and H-2 restricted. Induction was radioresistant, both on the part of the T cell and of the IL-1 producer, which was shown to be the APC, not the T cell. The IL-1 produced was the same in size, comitogenicity, and pyrogenicity as conventionally produced IL-1. The IL-1 induction process was attributable to a novel lymphokine produced by the T-cell clone and acting directly on purified macrophage and macrophage tumors to induce IL-1 production. This lymphokine was distinct from IFN-gamma, IL-2, and CSF-1 and may account for a principal mechanism of T APC signalling.

7. IL-2 Receptor-bearing T Cells in Unstimulated Spleen Cells from Unimmunized Mice: Localization to $L3T4^+$ Cells and Regulation by Non-H-2-linked Genes.

We made the surprising observation that IL-2 receptor-positive cells can be detected in fresh, unstimulated murine spleen T cells from unimmunized mice by flow cytometry using the monoclonal anti-receptor antibody 7D4. Also, unexpectedly, these cells were found exclusively in the $L3T4^+$ $Lyt2^-$ population by two-color fluorescence, in contrast to receptor-positive cells after stimulation, in which both $L3T4^+$ $Lyt2^-$ and $Lyt2^+$ $L3T4^-$ cells were found. The fraction of splenic T cells bearing IL-2 receptors reproducibly varies twofold under non-H-2-linked genetic control, with high expression in DBA/2 and BALB/c (about 6-7%) and low expression in B10.D2 and C57BL/6 (3%). This correlates quantitatively with a greater responsiveness of the DBA/2 and BALB/c splenic T cells to high doses of IL-2, compared to B10.D2 T cells. Twice as many B10.D2 T cells as DBA/2 T cells were required to get the same response. Studies with 23 BxD RI strains revealed that the level of IL-2 receptor-positive cells in unstimulated spleen cells was regulated by multiple genes, including very likely at least one gene on chromosome 7, near the HBB locus. The mapping makes novel use of nonparametric Smirnov statistics, which we suggest may be of general usefulness in similar analyses of RI strains.

8. Induction of Receptors for IgD on Cloned T cells by IgD and Interleukin-2 (with Richard Coico and Jeanette Thorbecke's laboratory).

The role of IgD in the immune response has remained elusive, although the predominance of IgD on the B-cell surface, and the paucity of IgD in serum have suggested a receptor function. In support of this hypothesis, it has recently been shown that receptors for IgD on helper T cells can be induced by exposure to IgD *in vivo* and *in vitro*. Such IgD receptor-positive T cells (i.e., T delta cells), detectable as rosette-forming cells (RFC) using IgD-coated sheep erythrocytes (SRBC), augment antibody responses. In this study, we showed that cloned, antigen-specific T cells of helper phenotype show only very low percentages of IgD-RFC if allowed to rest *in vitro* after antigen exposure in the absence of IL-2. Exposure to IgD or to IL-2 for 24 hours caused the IgD-specific RFC to increase as much as 25 fold to nearly 80%. Clones which have recently been stimulated with antigen, or T-cell hybridomas prepared from such clones, exhibited 40-50% IgD-RFC before exposure and two-fold higher levels after exposure to IgD. IL-2 also caused a

dose-dependent induction of IgD-RFC in normal splenic T cells. Thus, antigen stimulation, IL-2, and IgD can all induce these receptors for IgD, which presumably enable helper T cells to interact more effectively with delta^{+}B cells. The induction of IgD receptors by exposure of T-cell clones and cloned T hybridomas to IgD proves that: (1) the induction involves new expression of IgD receptors rather than expansion of a small T delta-positive population, and (2) the induction of IgD receptors is a direct effect of IgD on the helper T cell, not requiring a second (intermediary) cell.

9. Ir Gene Control of the Antibody and T-Cell Responses to a Malaria Circumsporozoite Vaccine (with Louis Miller's group).

Different H-2 congenic strains of mice were immunized with a P. falciparum sporozoite vaccine currently being tested in humans, or with different segments of the vaccine molecule. Specific IgG production or lymph node cell proliferation in response to different antigens was then determined. Only four of seven strains (representing three of eight possible different class II restriction molecules) responded to the vaccine. Of those restriction molecules, only one, I-A^{b}, was associated with a response to a malarial-encoded T epitope (contained within NP(NANP)$_3$NA), while the other two molecules ($E^{d}_{alpha}E^{d}_{beta}$ and $E^{k}_{alpha}E^{s}_{beta}$) were associated with a T-cell response to a non-malarial epitope(s) carboxyterminal to the malaria sequence and encoded by a tetracycline resistance gene, read out of frame. If an analogous situation applies in humans, natural boosting by sporozoites will be very restricted. This has serious implications for the effectiveness of the vaccine, since constant high levels of anti-sporozoite antibodies and possibly antibody-independent T-cell effector functions are required for immunity.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04021-03 MET

PERIOD COVERED

October 1, 1985 - September 30, 1986

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

Molecular Genetic Mechanism in Human Lymphoid Neoplasms

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

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John J. Wright	Medical Staff Fellow	MET, NCI
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David G. Poplack	Senior Investigator	DCT, PB
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Carolyn Felix	Medical Staff Fellow	POB

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	6	2

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☒ (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.)*

We noted that the t(14:18)(q32:q21) chromosomal translocation characteristic of follicular B cell lymphomas had a focused breakpoint at the 5' end of immunoglobulin (Ig) joining (J_H) segments and had extronucleotide "N" segments inserted at the chromosomal juncture. This indicates that Ig recombinase mediates the breakage on chromosome 14. However analysis of the breakpoint on chromosomal 18 reveals that a random staggered dsDNA break interupts an exon of a B cell associated gene. This gene is normally highly expressed in pre B cells but down-regulated with differentiation, but translocation results in an elevated amount of a fusion transcript with the Ig locus.

We've isolated cDNAs of the normal as well as translocated gene. We identified small major breakpoint region where over 70% of these translocation cluster and have used this marker of a transformation event to follow the clonal evolution of neoplasms and to refine cytogenetics of lymphomas.

We have further characterized the κ deleting element (Kde) which eliminates κ genes prior to λ light chain Ig use. We've found aberrantly rearranged V/J recombinants up stream of 75% of Kde rearrangements and noted that 75% of the time the Kde may rearrange directly with the V_K segment. We've noted duplication of the Kde and have chromosomally mapped related genes.

We extensively characterized the T Cell Receptor (TCR) and Ig gene in T as well as pre B form of acute lymphoblastic leukemia (ALL). Unique subsets of T cells were identified that were genetically indeterminant, or had unanticipated patterns of TCR α , β , γ gene rearrangement and expression. Moreover, a striking lineage spillover of γ TCR gene rearrangement was noted in pre B cell ALLs in contrast to mature B cells indicating they are a distinct subset of early B cell precursors.

GPO 914-918

Project Description

Major Findings:

The t(14;18)(q32;21) chromosomal translocation characteristic of over 80% of follicular B cell lymphomas was shown to be focused at the 5' end of immunoglobulin (Ig) joining (J_H) segments and to have extranucleotides "N" segments at the site of 14;18 juncture. This indicates that Ig recombinase mediates breakage on chromosome 14 and suggests that the t(14;18) occurs early in development at a pre B cell stage. A cosmid clone of the germline region of 18q21 was obtained and a 2.8 kb major breakpoint region (mbr) where over 70% of translocations occur was sequenced and most breaks clustered within but a 150 bp region. No conserved signals for recombinase were noted. Instead, the sequence analysis of both derivative 14 and 18 breakpoints revealed that the mechanisms of breakage on 18 was a random staggered dsDNA break and repair. Therefore, the focused breaks on 18 must be functional and we found that the breakpoint interupted an exon of a B cell associated gene at 18q21. This gene is expressed at high levels in pre B cells but down regulated with differentiation. In contrast the t(14;18) bearing mature B cell neoplasms have high levels of mRNA. The 18q21 gene is introduced head to tail with Ig heavy chain locus and a fusion transcript containing J_H and 18q21 information is generated. cDNAs of the translocated and non-translocated species of the 18q21 gene have been obtained. The 18q21 major breakpoint region serves as a molecular marker for the translocation and thus a transformation event enabling us to follow the natural history of these neoplasms. A single translocation is invariant over time in each lymphoma. However, variation in Ig genes on the normal chromosome 14 and of light chain genes occur and are a 2° clonal variation rather than true biclonality. We have also used the 18q21 major breakpoint region to refine lymphoma cytogenetics. Many 14q+ lymphomas (8/11) in which the reciprocal partner was indeterminant by routine cytogenetics were shown to be t(14;18) by gene rearrangement. We discovered that the normal chromosome 18 is prone to duplication and the der 18 is lost as part of a gene duplication event in such cells. In addition we've noted that some J_H rearrangements within T cell neoplasms can be classic 14;18 translocations indicating that just as D/J Ig rearrangements can spill over into T cells so can B cell type translocations.

We had identified and cloned a k deleting element (kde) that eliminated K genes prior to λ light chain gene expression. In further studies we've noted in 75% of instances the kde lands in the intervening sequence and proximal to this is an aberrant attempt at V/J_k joining. 25% of the time the kde specifically combines directly with the rearrangement signals flanking a V_k gene. We cloned and sequenced the germline form of the kde but can identify only a very short open reading frame that is evoluntionarily conserved with the mouse. We've noted that portions of the kde have been duplicated and three forms have been chromosomally mapped.

We have characterized the T cell receptor (TCR) and Ig genes within T and pre B cell Acute Lymphoblastic Lymphoma (ALL). Most T cell ALLs rearrange (22/25) and express their β TCR gene. Only the most mature T-ALLs express the α TCR and produce the complete T_3-Ti complex. Almost all (22/25) also rearrange the γ TCR gene whose function is as yet unknown, yet only a handful

still express the gene (3/15). Some leukemias with γ but neither β nor α activation were noted consistent with a hierarchy of γ before β before α. However, unique subgroups were found which had β but not γ rearrangement indicating the order is not invariant. Four examples of ALLs with totally germline TCR and Ig genes were noted. A unique T cell was identified with γ, α and T3 expression but no β rearrangement or expression. Considerably higher lineage spill over of γ TCR (40%) into pre B ALL was noted to 10% J_H Ig rearrangement in T ALL. This indicates that a subgroup of what had been termed pre B ALL are very early, rather genetically indeterminant with perhaps persistent recombinase activity. This series of studies utilizes the antigen specific receptor genes as molecular determinants of clonality, lineage, differentiation, and translocation.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04022-03 MET

PERIOD COVERED

October 1, 1985 through September 30, 1986

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

The Human Interleukin-2 Receptor

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

PI:	Warner C. Greene	Senior Investigator	MET, NCI
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	Mitchell Dukovich	Guest Worker	MET, NCI
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LAB/BRANCH

Metabolism Branch

SECTION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6		

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The human interleukin-2 (IL-2) receptor has been characterized at the gene, mRNA, and protein levels. The receptor gene contains 8 exons and is located on the short arm of chromosome 10. Transcription of receptor mRNA is initiated at two discrete start sites in normal activated T cells. The 5' flanking region of the receptor gene contains regulatory regions responsive to stimuli which activate IL-2 receptor expression as indicated by linking this region to the chloramphenicol acetyltransferase (CAT) reporter gene. Preliminary data also suggest the presence of an enhancer-like sequence located 5' to the two promoter segments. A seceted form of the IL-2 receptor protein has been produced using an "anchor-minus" cDNA construct expressed in mouse L cells. The full-length IL-2 receptor cDNA has also been expressed in human CEM T cells utilizing an amphotropic recombinant retroviral vector. The infected CEM T cells were proven to express both high and low affinity forms of the IL-2 receptor. The mechanism of HTLV-I- and HTLV-II-induced T-cell transformation and accompanying deregulated expression of IL-2 receptors has been explored. Utilizing retroviral vectors, the transactivator (*tat*) gene of HTLV-II was introduced into Jurkat T cells in both the sense and antisense orientation. These cells were then studied for altered expression of various cellular genes. We observed that the expression of genes encoding the IL-2 receptor, IL-2, and class II major histocompatibility antigens was activated in cells containing the sense *tat* gene but not in cells containing the antisense *tat* gene or infected with the control parental retrovirus lacking the *tat* gene. These data indicate that the transactivator gene of HTLV-I and -II not only augment viral replication but also alter the expression of genes which play a key role in T-cell activation and growth.

GPO 914-918

Project Description

Major Findings:

1. The IL-2 Receptor Gene

The human IL-2 receptor gene is organized as eight exons and seven introns spanning a distance of at least 25 kilobases (kb). In situ hybridization studies utilizing IL-2 receptor cDNAs radiolabeled with tritium by nick translation have localized the receptor gene to chromosome 10p band 14→15. The first exon of the receptor gene encodes the 5' untranslated region as well as the 21 amino acid signal peptide. The first intron is surprisingly large, encompassing at least 15 kb. Thus far, genomic phage clones bridging this intron have not been isolated, hence the precise length of the entire receptor gene is presently unknown and may be considerably greater than the minimal estimate of 25 kb provided. The N-terminal 66 amino acids of the mature receptor protein are encoded by the second exon which includes one of the two N-glycosylation sites (consensus sequence Asn-X-Ser/Thr). The third exon encodes the next 36 amino acids including the second N-glycosylation site. The fourth exon contains 72 amino acids which unexpectedly share considerable homology with the second exon. These data suggest that these exons were derived from an internal gene duplication event. As this region of internal homology is also present in the mouse IL-2 receptor cDNA, the internal duplication event must have occurred at least 50 million years ago, before the genetic radiation of mouse and man. The presence of these duplicated domains raises the interesting possibility, though presently unproven, that the IL-2 receptor may contain two ligand binding sites. The fourth exon also displays homology with the recognition domain of human complement factor B (Ba fragment). At present, the significance of this finding is unclear but suggests that these two proteins probably evolved from a common ancestral gene. The fourth exon infrequently is involved in aberrant post-transcriptional splicing, resulting in the joining of exon 3 and exon 5. When a cDNA corresponding to this aberrantly spliced mRNA is expressed in either COS-1 or L cells, it does not direct the display of functional membrane IL-2 receptors. The sixth exon contains multiple potential sites for O-linked glycosylation and, in the mature protein, encodes amino acids located immediately outside of the plasma membrane. A similarly positioned exon rich in O-linked glycosylation sites has been found in the low density lipoprotein (LDL) receptor. The seventh exon encodes the majority of the 19 residue hydrophobic transmembrane domain, while the eighth exon encodes part of the 13 amino acids comprising the intracytoplasmic domain as well as the 3' untranslated region of the IL-2 receptor. Located within the 3' untranslated region are at least three functional polyadenylation signal sequences as well as multiple Alu repetitive elements between the second and third poly A sites.

The 5' flanking region of the receptor gene contains important regulatory sequences involved in the control of IL-2 receptor gene expression. S1 nuclease protection and primer extension analyses have demonstrated the presence of two transcription initiation sites in normal activated T cells. "TATA-like" promoter sequences are present 25-30 bp upstream from each of these initiation sites which are separated by 58 nucleotides. This 5' flanking region has functional promoter activity as evidenced by its capacity to direct the expression of the chloramphenicol acetyl transferase (CAT) gene following transfection into Jurkat T cells. These two transcription initiation sites are equivalently utilized in normal activated T

cells. Furthermore, these upstream sequences are responsive to activation signals which increase IL-2 receptor expression. Finally, an enhancer element may also be present in this region.

2. Multiple IL-2 Receptor mRNAs

Three discrete size classes of IL-2 receptor mRNA have been detected by Northern blot analyses. S1 nuclease protection studies demonstrated that these differently sized mRNAs are generated by the use of three different polyadenylation signal sequences located within the 3' untranslated region of the IL-2 receptor gene. Suboptimal stimulation of normal T cells appears to result in preferential use of the most 3' poly A site, while full stimulation produces more equivalent use of each of the three sites. At present, it is unknown whether certain receptor mRNA species are more stable or more efficiently translated than others. The combination of two transcription initiation sites, the variable post-transcriptional splicing of exon 4, and the three poly A sites indicates that as many as 12 discrete IL-2 receptor mRNAs may exist in activated normal T cells.

Following PHA activation of peripheral blood lymphocytes, mature IL-2 receptor mRNA is detectable within 4 to 8 hours. Within 2 hours, a larger, possibly nuclear precursor RNA species is produced which disappears as the mature receptor mRNA appears. IL-2 receptor mRNA levels peak at 8 to 24 hours and then decline. Nuclear runoff assays demonstrate that the rise and fall in whole cell IL-2 receptor mRNA levels is produced by changes in the transcriptional activity of the IL-2 receptor gene. Interestingly, whole cell mRNA levels appear to decline somewhat earlier than the fall in receptor gene transcription, suggesting that receptor expression may also be controlled at a post-transcriptional level.

3. IL-2 Receptor Protein

The amino acid sequence of the human IL-2 receptor has been deduced by DNA sequence analysis of a full-length IL-2 receptor cDNA. The mature receptor protein is composed of 251 amino acids. The primary translation product contains an additional 21 residues comprising the signal peptide. Thirteen cysteine residues are present within the receptor, some of which participate in the formation of intrachain disulfide bonds. Interchain disulfide bonding has not been detected. The receptor contains two sites for N-linked glycosylation and multiple sites which may be involved in O-linked sugar addition. A 19 residue, hydrophobic region forming the putative transmembrane domain is present near the carboxy terminus of the protein. In view of the position of the signal peptide and N-linked carbohydrate addition sites, the receptor is most likely an NH_2 terminus out, COOH terminus in transmembrane structure. An unexpected feature of the IL-2 receptor is that its intracytoplasmic domain is composed of only 13 amino acids. Thus, unlike many other growth factor receptors, this intracytoplasmic domain is insufficient in length to encode an enzymatic activity, in particular a tyrosine kinase. The region, however, does exhibit features of an intracellular domain including the presence of positively charged amino acids which probably function to anchor the receptor protein within the plasma membrane. This anchoring function of this domain has been confirmed by the introduction of a translation termination codon in the cDNA following the fourth residue of the transmembrane domain. When expressed in mouse fibroblasts, this truncated IL-2 receptor cDNA lacking the intracytoplasmic domain

exclusively directed the synthesis of a secreted form of the IL-2 receptor which retained the capacity to bind IL-2 and anti-Tac.

The sequential steps in IL-2 receptor biosynthesis and post-translational processing have also been defined. The primary translation product (M_r of 34,500) was identified by translation of normal activated T-cell RNA in a cell-free wheat germ lysate system and immunoprecipitation with a rabbit polyclonal anti-IL-2 receptor antiserum. Pulse-chase labeling studies performed with [^{35}S]methionine, in the presence of tunicamycin to block N-glycosylation, permitted identification of the unmodified mature receptor backbone as a 33,000 M_r protein. The size difference of this precursor and the primary translation product is consistent with cotranslational cleavage of the 21 residue signal peptide. Pulse-chase labeling without tunicamycin permitted identification of two additional precursor proteins with M_rs of 35,000 and 37,000. Digestion of these precursor proteins with endoglycosidase F, which removes N-linked sugar, converted each to the 33,000 M_r form. These data indicated that the IL-2 receptor peptide backbone is cotranslationally modified by addition of N-linked carbohydrate addition, producing two intermediately sized precursors. Following 30 to 60 minutes of chase, the precursor proteins disappeared as the 55,000 M_r mature receptor form appeared. Mature receptor production was blocked by addition of the carboxylic ionophore, monensin, known to interfere with Golgi-associated protein processing and transport. Further enzymatic digestion study of the mature receptor protein confirmed the presence of O-linked carbohydrate and sialic acid, suggesting that these sugar moieties were added during passage through the Golgi apparatus.

The IL-2 receptor is also modified by post-translational addition of sulfate. The site of sulfation remains undefined as well as the role played by this form of receptor modification. The IL-2 receptor also is constitutively phosphorylated, principally involving serine 247 within the intracytoplasmic domain. Thus far, data demonstrating IL-2-induced phosphorylation of its receptor has not been obtained although additional study of this possibility is needed.

4. High and Low Affinity IL-2 Receptors

The early IL-2 receptor binding studies performed by Robb and associates with purified radiolabeled IL-2 prepared from induced Jurkat T cells revealed 2,000-4,000 receptors per PHA-activated T lymphoblast. Scatchard analysis of these binding data indicated a Kd of 2-10 pM. In contrast, binding studies with the same cells utilizing radiolabeled anti-Tac antibody indicated 30,000 to 60,000 IL-2 receptors per cell. Scatchard plots of antibody binding suggested a single affinity class of receptors. The apparent difference in receptor number was not due to Fc receptor binding by anti-Tac nor caused by partial occupancy of receptor sites by endogenously produced IL-2. These data suggested the possibility that the high affinity IL-2 binding studies were detecting only a subgroup of the IL-2 receptors. To test the possible existence of a low affinity class of IL-2 binding sites, the IL-2 binding studies were repeated employing much larger quantities of ligand. These assays revealed a previously undetected large pool of IL-2 binding sites displaying a 1,000- to 10,000-fold lower apparent affinity for ligand (Kd of 20-40 nM). The sum of the high and low affinity IL-2 binding sites was nearly equivalent (within a factor of 2) to the number of sites measured with anti-Tac. These data suggested that anti-Tac interacts with both the high and low affinity forms of the IL-2 receptor. Furthermore, only 5-10% of the membrane IL-2 recep-

tors existed as high affinity sites; however, these receptors appear to mediate the growth-promoting response to IL-2. At present, the function of the more numerous low affinity IL-2 receptors remains undefined. Utilizing ^{125}I-IL-2 binding under high and low affinity conditions, Weissman and colleagues have recently demonstrated that the high affinity IL-2 receptors undergo receptor-mediated endocytosis. In contrast, the low affinity receptor class is not internalized after ligand binding. These findings further underscore a basic functional and structural difference in these receptor classes.

The molecular difference in high and low affinity receptor structure is not yet known. Three possibilities, however, seem plausible. First, the high affinity IL-2 receptor may be produced by the assembly of a receptor complex with one or more other proteins. This model is attractive in terms of explaining growth signal transduction through the high affinity receptor. Since the intracytoplasmic domain of the IL-2 binding protein is only 13 amino acids in length, it is difficult to envision how such a short domain mediates effective signaling for T-cell proliferation. It is conceivable that the other putative protein(s) involved in the high affinity IL-2 receptor complex may not only alter receptor affinity for ligand but also provide critical components involved in signal transmission. A second consideration is that the high affinity IL-2 receptor may be produced by a particular post-translational processing event. This model, however, does not provide a convenient explanation for signal transduction. A third possibility which has been suggested is that the high and low affinity forms of the IL-2 receptor are, in fact, different proteins encoded by different genes. In this regard, expression of the human IL-2 receptor cDNA in mouse fibroblasts results in the expression of only low affinity receptors. However, these findings could also reflect the absence in L cells of the companion proteins required for receptor complex formation or, alternatively, a failure of appropriate post-translational processing in nonlymphoid cells. Taniguchi and colleagues have recently achieved high affinity IL-2 receptor expression in mouse T cells following introduction of human IL-2 receptor cDNA. Similarly, we have reconstituted functional high affinity IL-2 receptors in human T cells by infection of these cells with an amphotropic retrovirus containing the IL-2 receptor cDNA. Thus the model involving one gene for the high affinity receptor and a second gene for the low affinity receptor is incorrect. These data do not, however, distinguish between the receptor complex versus post-translational processing models for high affinity IL-2 receptor expression.

5. Deregulated IL-2 Receptor Expression in HTLV-I-Induced Adult T-Cell Leukemia

HTLV-I infection of human T4+ lymphocytes can result in the adult T-cell leukemia (ATL). This aggressive and usually fatal leukemia often produces a clinical syndrome of hypercalcemia, dermal or epidermal leukemic infiltrates, and increased susceptibility to opportunistic infections. Cases of ATL are geographically clustered in areas where HTLV-I is endemic, including the southwestern region of Japan, the Caribbean basin, the southeastern United States, and sub-Saharan Africa. Thus far, an effective therapy has not been defined. Cultured ATL cell lines uniformly display large numbers of membrane receptors for IL-2 involving both high and low affinity sites. However, most of these long-term ATL cell lines, while displaying IL-2 receptors, do not produce IL-2 mRNA or protein nor require IL-2 for growth.

The constitutive expression of IL-2 receptors in ATL cell lines does not appear to involve chromosomal translocation, rearrangement, or amplification of the IL-2 receptor gene. The primary amino acid sequence of the normal and leukemic receptor also appears to be identical. ATL cell lines constitutively express large quantities of IL-2 receptor mRNA and each of the three known poly A addition sites are used. Furthermore, post-transcriptional splicing of exon 4 have been identified in normal and HTLV-I-infected T cells. Nuclear runoff assays confirm that the IL-2 receptor gene is transcribed at a high level in the ATL cells.

Comparison of IL-2 receptor promoter structure in normal and ATL cells has revealed an interesting difference. As noted above, IL-2 receptor gene transcription in activated normal T cells is initiated at two principal sites. In contrast, while also using these two sites, ATL cells initiate transcription from a third additional site located further upstream. The presence of a new transcription start site in these HTLV-I-infected T cells is intriguing in view of high level receptor expression; however, the third promoter appears to be considerably weaker than the two promoters shared by normal and ATL cells. While it is conceivable that the mRNAs generated from the third transcription initiation site are more stable or more efficiently translated, it seems unlikely that this extra promoter provides the full explanation for the high level expression of IL-2 receptors encountered in this leukemia.

HTLV-I and HTLV-II, while able to acutely transform human cord blood T cells, do not contain a recognized oncogene characteristically present in other acutely transforming animal retroviruses. Furthermore, these viruses do not appear to transform T cells by promoter-enhancer insertion with cis-activation of an endogenous cellular oncogene since the sites of proviral integration vary from tumor to tumor. The HTLV-I provirus, like other retroviruses, contains flanking long terminal repeats (LTRs) and gag, pol, and env genes. However, an extra region, termed pX, located near the 3' end of the virus, was also identified. Sodroski, Rosen, and Haseltine subsequently demonstrated that the pX region encodes a protein following a unique double splicing event which is capable of activating in trans the transcription of genes controlled by the viral LTR. This trans-activator gene was originally termed LOR or X-LOR but more recently tat (transactivator of transcription), given its function. It has been suggested that the tat protein may, in addition to augmenting viral transcription, also activate the expression of select cellular genes involved in T-cell growth. To test this hypothesis, the tat gene of HTLV-II was inserted into a retroviral expression vector and used to infect Jurkat T cells and Raji B cells following packaging in psi AM cells. For controls, Jurkat and Raji cells were infected with viruses containing the tat-II gene in the anti-sense orientation or the parental ZIP-NEO virus. Following G418 antibiotic selection for the presence of the neomycin resistance gene, cells were evaluated for the production of functional tat-II protein. Jurkat and Raji cells containing the sense, but not anti-sense tat-II gene were found to produce a 38,000 dalton protein which trans-activated HTLV-I LTR-CAT plasmids while not altering pSV2S CAT expression. These cell lines were then used to evaluate potential changes in cellular gene expression. Northern blot analyses of poly A+ RNA demonstrated the presence of IL-2 receptor mRNA in the Jurkat T cells containing the tat-II gene in the sense orientation. In contrast, IL-2 receptor mRNA was not detected in the Jurkat T cells infected with the ZIP-NEO virus or in Jurkat T cells containing the anti-sense tat-II gene. In contrast to Jurkat T cells, introduction of the tat-II gene into Raji B cells

did not induce activation of IL-2 receptor expression. S1 nuclease mapping of the transcription initiation sites for IL-2 receptor expression in the Jurkat-tat-II cells revealed exclusive use of the upstream start site unique to HTLV-I ATL infected cells.

IL-2 receptor protein was also detectable in the Jurkat-tat-II T cells by indirect immunofluorescent staining, immunoprecipitation, and [^{3}H]anti-Tac binding assays. These latter binding studies revealed that the Jurkat-tat-II cells contained only 1,000 to 1,500 IL-2 receptor sites per cell in contrast to HTLV-I-infected HUT 102-B2 which display more than 100,000 sites per cell. This quantitative difference in the level of IL-2 receptor expression may reflect intrinsic properties of the parental Jurkat T cells used for infection or be due to the fact that low levels of tat-II protein are produced. The retroviral vector used for the expression of the tat-II gene in these studies contains a Moloney virus LTR which is not susceptible to trans-activation by the tat-II protein. Thus, unlike the HTLV-I or -II provirus where the tat protein trans-activates its own production, tat does not augment its expression in the ZIP-NEO retroviral vector. Immunoprecipitation analysis has confirmed that the Jurkat-tat-II cells contain considerably less tat-II protein than HTLV-I-infected ATL cell lines. It is possible that high levels of the tat protein are required for full activation of IL-2 receptor expression.

Activation of other genes involved in T-cell growth by tat-II was also studied. Interestingly, the tat-II protein induced IL-2 mRNA protein expression in Jurkat T cells but not in Raji B cells. These findings suggest a possible scenario whereby the tat-II protein subverts normal growth control in T cells by stimulating autocrine proliferation involving IL-2 and its cellular receptor. However, the majority of HTLV-I-infected ATL cell lines, as well as freshly isolated tumor cells from ATL patients, do not secrete IL-2 nor contain detectable levels of IL-2 mRNA. Furthermore, most long-term cultured ATL cell lines are not dependent on IL-2 for growth. We surmise that these findings may represent natural tumor progression from an early growth factor-dependent stage to a later growth factor-independent stage. Similarly, other tumor systems have been found to exhibit early dependence on growth factors which later is replaced by growth factor autonomy. The biochemical event(s) leading to clonal proliferation, growth factor independence, and full transformation remain undefined at present. It seems likely that the tat gene may mediate the first step in this process but be insufficient to complete the transformation process.

Retroviral-induced tumors have now been associated with the introduction of oncogenes resembling growth factors (v-sis --> platelet-derived growth factor) or growth factor receptors (v-erb-B -> epidermal growth factor receptor). Alternatively, retroviruses may produce leukemia by cis-activation of an endogenous cellular oncogene via promoter-enhancer insertion (e.g., avian leukosis virus activation of c-myc expression). The HTLV-I and -II retroviruses appear to employ a third unique strategy involving their trans-activator gene which results in the inappropriate expression of host cellular genes encoding IL-2 and the IL-2 receptor.

6. Summary

The human IL-2 receptor has now been characterized at the gene, mRNA, and protein levels. It is clear from these studies that expression of this receptor is a critical event in the growth of normal T cells and may be grossly altered in HTLV-I or -II retroviral-induced leukemias. The availability of cloned genes for both the IL-2 receptor and IL-2 make this system attractive for further study. Future studies will likely focus on (1) the molecular differences which distinguish high and low affinity IL-2 receptors, (2) the control of IL-2 receptor gene expression, (3) the mechanism of IL-2 signal transduction, and (4) the trans-activator genes of HTLV-I and -II and their relationship to activation of IL-2 and IL-2 receptor gene expression and the potential role played by these genes in leukemic transformation.

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SUMMARY REPORT
IMMUNOLOGY BRANCH
October 1985 - September 1986

The Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis in the following areas: 1) Regulation and control of immune responses; 2) Structure and function of cell surface molecules; 3) Transplantation biology; 4) Molecular biology and 5) Tumor immunology including clinical studies in immunotherapy. In addition, the Immunology Branch maintains a fluorescence activated cell sorter facility which is involved integrally in many of the studies carried out in the Branch in each of the above areas, and is also used in a large number of collaborative investigations with other laboratories at NIH. This report summarizes research efforts in each of these areas during the past year. More detailed information on specific accomplishments can be found in the individual annual reports.

A. REGULATION AND CONTROL OF IMMUNE RESPONSES

Recent studies from Dr. Singer's laboratory have demonstrated that the selection of $L3T4^+$ T cells specific for composite class I + class II MHC determinants is regulated by both class I and class II MHC genes (5064). This is the first demonstration of gene complementation between class I and class II MHC genes, and suggests a novel mechanism of T cell selection and immune response gene regulation in which T cells are selected during ontogeny on the basis of their specificity for composites of self-MHC and self-antigens.

Studies from Dr. Singer's laboratory have also demonstrated that the generation of class II-restricted CTL is actively downregulated by class I restricted regulatory T cells (5111). Thus, class II-restricted CTL are generated in response to defined class II antigens, but are suppressed in responses involving complex antigenic challenge.

Dr. Hodes' lab has studied the ability of different Ia expressing populations to present antigen to cloned T cells. All of the clones tested were able to respond to macrophage/dendritic cell-containing populations or to purified resting B cells. In contrast, only a subpopulation of cloned T cells was responsive to LPS activated B cell blasts, in spite of enhanced Ia expression by these B cell blasts. These findings indicate that the signals required for T cell activation may vary among T cell clones, and that distinct Ia bearing antigen presenting cells may be competent for presentation to different T cells (5069). In addition the activation requirements of cloned and naive T cells has been evaluated by analyzing activation induced by monoclonal antibody to the T cell receptor structure. Naive $Lyt2^+$ T cells were activated to proliferate by soluble anti receptor antibody in the presence of IL-2 and accessory cells, whereas $L3T4^+$ naive T cells were unresponsive under the same conditions. Specific "targeting" of the T cell receptor to accessory cell structures by heteroaggregates of monoclonal antibodies directed to cell surface determinants was, in contrast, capable of activating both T cell subpopulations. These studies provide a model system for further assessing the activation requirements for receptor-mediated triggering of resting and activated T cells (9205).

Collaborative studies involving Dr. Hodes' and Dr. Sachs' labs have been directed toward analysis of the genetic regulation in T cell responses to staphylococcal nuclease (NASE), a series of cloned T cell lines were generated. Individual clones were restricted to recognizing NASE in the context of unique Ia molecules, and the antigen fine specificity of these T cells was identified through the use of 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. 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, and provide further information critical to the understanding of MHC restricted antigen presentation (5086).

A series of monoclonal antibodies to nuclease have also been developed (5036), and have been used in kinetic inhibition studies to determine the mechanism of inhibition of catalytic activity by this enzyme. The antibodies have been divided into at least three groups according to their epitope binding, as assessed by competitive inhibition of ELISA.

The role of T cells in regulating the fine specificity of idiotype dominant responses to phosphocholine and azobenzene arsonate was studied. It was found that cloned populations of carrier specific and MHC restricted T helper cells were capable of supporting idiotype dominant responses in B cells of appropriate haplotype for both of these responses. These findings demonstrated that no absolute requirement exists for the participation of idiotype specific T helper cells in the generation of optimally idiotype dominant responses in these experimental systems (5108).

Dr. Shaw's studies of conjugate formation between cytotoxic cells and their targets identify two pathways of antigen-independent conjugate formation: one pathway mediated by CD2 binding to LFA-3 and the other mediated by CD18 presumably by binding to an undefined ligand (5067). His studies suggest that antigen-independent conjugate formation is an essential step which can occur independently of (and perhaps precede) antigen-specific recognition.

Recent studies in Dr. Dickler's laboratory have revealed that activated B lymphocytes significantly augment responses of resting B lymphocytes via a mechanism involving two components: one membrane-bound and one soluble (9201). This observation bears on our understanding of B lymphocyte growth and maturation.

Dr. Ting has shown that generation of CTL is regulated by lymphokines. In addition to interleukin 2 (IL2), T cell differentiation factor (TCDF) also regulates the CTL responses to mitogens and alloantigens. TCDF production requires both L3T4 cells and macrophages, and is regulated by self Ia molecules (5118).

Dr. Shearer's laboratory has found that the selective loss, by lymphocytes from HTLV-III/LAV seropositive individuals, of T cell immune responsiveness to influenza virus but not to major histocompatibility transplantation antigens is an early functional indicator that immune dysfunction has occurred that will lead to AIDS-like symptoms. This observation may be one of the earliest signs of onset of AIDS. The study indicates that multiple helper T cell subsets can be identified in human—only one of which is the primary target of the retrovirus (5110).

In addition activated T cells infected in vitro with HTLV-III/LAV have been shown to produce more virus than resting cells. The type of antigenic stimulus used for T cell activation is important in the survival of the T cell cultures and in the kinetics of virus production. It is postulated that the onset of symptomatic AIDS and the course of the disease can be affected by the types of antigenic stimuli that HTLV-III/LAV-infected individuals encounter (5110).

Dr. Shearer's laboratory has also observed that a graft-versus-host reaction induced by class II MHC recognition results in the selective loss of the $L3T4^+$ but not of the $Lyt2^+$ T helper cell pathway. This selective loss of function by one T helper subset mechanistically resembles the developmental stages of AIDS. It also is demonstrable in graft-versus-host examples that are associated with autoimmunity, and may serve as a model for T cell dysfunction in autoimmune diseases (5088). It has also been shown in the mouse model that animals undergoing a graft-versus-host reaction lose not only peripheral immune function, but they also exhibit severe stem cell defects, and the ability of the thymus from GVH mice to develope self-restricted T cell immune responses is destroyed (5088).

Studies in Dr. Segal's lab have shown that human peripheral blood T cells can be retargeted, in vitro, against most cell types by coating them with anti-T3 cross-linked to anti-target cell antibodies. The effector cells are $T8^+$ and are activated by brief exposure to recombinant human IL-2 (5050). Similarly, human K cells can be retargeted with anti-FcR crosslinked to anti-target cell antibody. In this case the effector cells are $Leu11^+$, $T3^-$, and partially (about 30%) $T8^+$. These same cells mediate NK, which is greatly enhanced relative to the antibody-dependent lysis, by recombinant IL-2. These studies suggest that cell mediated cytotoxic activities could be artifically manipulated to turn the immune system against unwanted cells, for example, tumor cells (5050).

B. STRUCTURE AND FUNCTION OF CELL SURFACE MOLECULES

Recent studies in Dr. Bluestone's laboratory on the analysis of cell surface antigens on T cells and their role in T cell activation and recognition have shown that anti Ly 6 antibodies as well as anti T cell receptor antibodies can be used to activate cytotoxic T cells and proliferation of resting T cells. These findings suggest that the Ly 6 molecule as well as the T cell receptor complex play an important role in T cell activation (5112).

Recent studies in Dr. Bluestone's laboratory on the structure function relationship of MHC class I molecules has shown that single amino acid changes result in profound effects on T cell recognition. These findings support the concept that the MHC molecule structure is susceptible to small perturbations which effect allo recognition (5117).

Recent studies in Dr. Dickler's laboratory indicate that down-regulation of B lymphocyte differentiation occurs in response to a signal cooperatively generated by two distinct membrane receptors (FcλR and IgM) each occupied by its respective ligand, and that B lymphocytes from certain strains of auto-immune mice do not respond to this downregulatory signal (5035). These findings suggest that FcλR play a role in regulation of humoral immune responses. In addition, Recent work in Dr. Dickler's laboratory has resulted in production of a monoclonal antibody which reacts primarily with a membrane

molecule on activated B lymphocytes which appears to be previously undescribed (9200). This molecule may be a lymphokine receptor.

Dr. Gress has found evidence that the ligand for T8 is not a class I molecule, that anti-T8 does not mediate blocking by providing an "off-signal", that LFA2 is likely part of the signal sequence for cytotoxic T cell activation, and that such cytotoxic T cells rely entirely on their LFA1 and LFA2 cell surface molecules for interacting with target cells (5116).

One approach used in the laboratory of Dr. Shaw to analyze CTL recognition of DPw2 has been the generation of mutant lymphoblastoid cell lines which have lost the capacity to be recognized by DPw2-specific CTL. Molecular analysis of these mutants is in progress and suggest that the limited polymorphism of the DPα chain (between DPw2 and DPw4) is nevertheless critical for T cell recognition (5101).

Recent studies from Dr. Singer's laboratory on the role of L3T4 accessory molecules in T cell interactions have indicated that, if the L3T4 molecule binds to determinants on the Ia molecule, those determinants must be encoded within the highly polymorphic external domains of Ia rather than the less polymorphic membrane-proximal domains of Ia (9204). Other studies from Dr. Singer's laboratory on the role of Lyt2 accessory molecules indicate that anti-Lyt2 antibodies can actively modulate the function of $Lyt2^+$ T cells by a mechanism that has not yet been fully elucidated (9204).

Studies on the biochemistry of cytoplasmic granules of large granular lymphocyte tumor cells in Dr. Henkart's lab have resulted in the purification of two distinct trypsin-like enzymes which are major granule components. These studies aid in our understanding of the mechanism of NK cell cytotoxicity (5018). In addition, studies on the mechanism of cytotoxic T lymphocyte killing carried out in Dr. Henkart's laboratory indicate that serine esterases in killer cell cytoplasmic granules play a vital functional role in the cytolytic process. These observations support the granule exocytosis model as the basic explanation for the mechanism of lymphocyte cytotoxicity (5103).

C. TRANSPLANTATION BIOLOGY

Studies in Dr. Sachs' laboratory have been directed toward understanding the structure and function of products of a major histocompatibility complex and manipulations of the immune response to these products. A large series of monoclonal antibodies to H-2 and Ia antigens have been produced and have been used both to study the complexity of MHC antigens and to develop anti-idiotypic reagents reactive with anti-MHC receptors (5021). Analysis of cell surface antigens using these monoclonal antibodies has demonstrated two new H-2 products determined by genes within the D region.

In addition, Dr. Sachs and colleagues have reported a new method for inducing transplantation tolerance by reconstituting irradiated animals with mixtures of syngeneic and allogeneic bone marrow (5021). Mixed chimeras produced in this fashion have been found to be fully tolerant to syngeneic and allogeneic skin grafts and to be fully immunocompetent. In vitro assays designed to examine the mechanism of this tolerance have shown the presence of nonspecific suppressor cells in the early weeks following reconstitution (5021). These suppressor

cells are not T cells, are radiation sensitive, and may be involved in the first phase of elimination of alloreactive cells. Attempts to extend this mixed marrow tolerance induction to a larger animal model have been initiated using partially inbred miniature swine (5023).

Other studies of transplantation biology in the miniature swine model have been continued. 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 (5023). Class II matching has been found to be much more important than class I matching in determining the survival of such allografts. Acceptance of vascular allografts has also been demonstrated to induce systemic tolerance (5023). In vitro studies of this tolerance indicate an absence of precursor cells capable of mounting a CTL response across a class I only difference. A series of monoclonal antibodies reactive with a variety of swine lymphocyte surface antigens has been prepared (5023). These antibodies are being used to study cell surface antigens in swine lymphocyte populations, as well as for T cell depletion in transplant studies.

Studies from Dr. Singer's laboratory have identified the T cell subsets involved in the rejection of skin allografts expressing various antigenic disparities. Notably, their studies have demonstrated that $Lyt2^+$ T cells with helper function are the critical cells in initiating the rejection of class I MHC disparate skin grafts (5119). In addition, their studies suggest that collaboration between phenotypically distinct T cell subsets is critical for the rejection of skin allografts expressing non-MHC antigens but is not a feature of the rejection of skin allografts expressing MHC antigens (5122). Finally, recent studies from Dr. Singer's laboratory have indicated that the cells in the skin which express class II MHC antigens express very little if any class I MHC antigens, and vice versa (5122). This previously unappreciated skewing of MHC antigen expression importantly influences the specificity and function of the T cell subsets that can be involved in skin allograft rejection.

Studies by Dr. Gress and colleagues with T cell depleted autologous bone marrow in rhesus monkeys showed that low numbers of residual T cells ($\leq$0.008%) could be detected by a limiting dilution assay, that residual T cell content correlated with early graft rejection, that recovery of the $T4^+$ subset in the reconstituted animals is prolonged whether the animals received T cell depleted or non-T cell depleted marrow, that $T8^+$ cells recover early during reconstitution, that graft rejection correlates best with the presence of $T4^+$ cells in the setting of a functional $T8^+$ subset, and that graft survival is significantly prolonged (without further immunosuppression) in animals receiving T cell depleted marrow (5116).

The minor lymphocyte stimulating (Mls) determinants represent the only antigenic determinants aside from those encoded in the major histocompatibility complex which are capable of stimulating T cell recognition at high frequency, as reflected in primary proliferative responses of naive T cells. The nature of polymorphism and allelism in this genetic system has remained poorly defined to the present time. A series of T cell clones specific for Mls^a, Mls^c, and Mls^d has been generated and these clones used to define the polymorphism and allelism of Mls determinants. Findings from these studies indicate that the Mls system as currently defined is not an allelic single locus system, but represents the products of at least two distinct and unlinked genes (5106).

Dr. Shearer's laboratory has developed a model of interstitial pneumonitis that involves the synergistic effects of murine cytomegalovirus infection and graft-versus-host reaction. In such animals, large numbers of donor-anti-host T cells infiltrated the lungs of the recipients that was not observed when either insult was given alone. These results could be of relevance for understanding the contributing factors involved in the appearance of interstitial pneumonitis in transplant situations (5099 and Contract NO1-CB-51014).

D. MOLECULAR BIOLOGY

The studies in Dr. Dinah Singer's lab have centered on the regulation of expression (5124, 5115, 9203) and genomic organization (5114, 5123) of the class I MHC multigene family. In the miniature swine, unlike other species, the family consists of only seven members. Analysis of isolated class I genes reveals distinct regulatory mechanisms controlling their expression in vivo (5115, 5123). Thus both quantitative and qualitative differences in the patterns of expression are observed. Development of in vitro model systems, such as transfection and transgenic mice, has allowed for detailed molecular analysis (5115, 5124). Thus far, analysis of one of these genes has defined not only the promoter, but also positive and negative regulatory elements (5115). Structural analysis of the class I MHC genes has revealed that although these genes may be differentially expressed, they share common structures (5114). Homologies within the family range from 85% to 60%. Although a variety of single nucleotide changes are observed in the exons of these genes, complex alterations are also seen, consistent with the interpretation that some mechanism of gene conversion may operate to generate polymorphism in this family. In the mouse, a novel class I MHC gene has been identified and characterized. This gene, which is highly divergent from other members of the family, may represent a primordial gene which antedates speciation (9203).

Efforts to study and to clone the genes responsible for class II antigens in miniature swine have been begun in Dr. Sachs' laboratory. Southern blot analyses of lymphocyte DNA from each of the miniature swine herds and recombinant lines have been performed. The data indicate that these swine possess single class II alpha chain genes corresponding to those of the human DR alpha chain, DQ alpha chain, and DP alpha chain. Of these, only the DQ alpha chain gene appears to be polymorphic within these herds. Approximately six class II beta chains have been revealed by these analyses and show extensive cross hybridization between beta chains of the three major loci (5023). A complete phage library has been constructed from the genomic DNA of the C haplotype, and screening is in progress for isolation of class II genes. This library complements the cosmid library from the D haplotype which has previously been produced in Dr. Dinah Singer's laboratory (5114).

Cloning of the LFA-3 gene is being undertaken in the laboratory of Dr. Shaw both by "shotgun" transfection and by oligonucleotide probing of a cDNA library after determination of the protein sequence of LFA-3 (5067).

Research in Dr. Kelly's laboratory has shown that proto-oncogenes encoding the nuclear-localized proteins, c-fos, c-myc, and c-myb, are transcriptionally regulated by signals transduced following mitogen binding to the surface of lymphocytes. Because oncogenes appear to be closely associated with tumor formation, these studies begin to define gene products that may play a role in the regulation of lymphocyte proliferation. In order to identify additional genes that are

regulated in parallel with the above oncogenes, a cDNA library that is greater than ten-fold enriched for such sequences has been constructed (5120).

E. TUMOR IMMUNOLOGY

When either anti-T3 or anti-FcR are crosslinked with monoclonal anti-tumor antibodies, they cause fresh human peripheral blood T or K cells to specifically lyse tumor targets. Dr. Segal and colleagues have now shown that, in vitro, human T cells coated with anti-T3 crosslinked to anti-tumor specifically lyse fresh tumor target cells but not fresh cells prepared from normal tissue (5050). In Winn type tumor neutralization assays in nude mice, human peripheral blood T or K cells have potent activity against human tumors when coated with the appropriate crosslinked antibodies. These studies demonstrate that such effector cells can specifically prevent tumor growth in an in vivo environment and justify further studies on the destruction of established tumors in vivo (5050).

The cellular basis for in vitro generation of activated killer cells against tumors has been partially established in a mouse model in Dr. Wunderlich's lab. Polyinosinic acid (Poly I) and purified recombinant IL-2 were used as the activating agents (5003). Precursor cells, which include both Thy 1^+ and Thy 1^- cells are stimulated by IL-2 and probably by additional lymphokines produced by accessory cells and $L3T4^+$ helper T cells. The generation of anti-tumor cytotoxic cells is inhibited in a strain-dependent fashion by accessory cells. In addition, Dr. Ting has shown that Lymphokine activated killer cells (LAK) are induced by culturing normal spleen cells with syngeneic macrophages and indomethacin, or by culturing normal spleen cells with exogenous IL2. The LAK precursors are Asialo $GM1^+$, $Thy1^-$ and $Lyt2^-$ cells, and the LAK effectors are Asialo $GM1^-$, $Thy1^+$ and Lyt2 cells. During the differentiation of the LAK precursors into LAK effectors, the Asialo GM1 expression decreases and the Thy1 expression increases (5118).

F. FLUORESCENCE ACTIVATED CELL SORTER

The Immunology Branch has continued to maintain an active Fluorescence Activated Cell Sorter Facility operated by Ms. Susan Sharrow and under the guidance of Dr. J. Wunderlich. The Fluorescence Activated Cell Sorter (FACS) has been used both to analyze and to separate lymphoid populations by rapid flow microfluorometry. Studies performed in this facility have been an integral part of many of the investigations described in the above sections, as well as of numerous collaborative studies with other investigators at NIH and elsewhere.

The flow cytometry laboratory has supported a variety of projects by analyzing and separating cells based on the quantitative expression of various cell-surface determinants some of which have been summarized (5062). Within the last year, the laboratory has helped analyze 1) the basis for T-lymphocyte recognition of transplantation antigens, 2) the regulation of IL-2 receptors and cells expressing these receptors (which are critical for T-lymphocyte differentiation), 3) the quality of gene-transfection of cells, and 4) the function of immune cells in the skin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05003-21 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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:	J. R. Wunderlich	Senior Investigator	IB, NCI
Others:	C. C. Ting	Medical Officer	IB, NCI
	D. Segal	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

The cellular basis for in vitro generation of activated killer cells against tumors has been partially established, using polyinosinic acid (Poly I) and purified recombinant IL-2 as the activating agents. Precursor cells, which include both Thy 1^+ and Thy 1^- cells are stimulated by IL-2 and probably by additional lymphokines produced by accessory (macrophage and/or B cells) cells and $L3T4^+$ helper T cells. The generation of anti-tumor cytotoxic cells is inhibited in a strain-dependent fashion by accessory cells. Together, low doses of IL-2 and ancillary activating agents (e.g., Poly I) generate levels of anti-tumor activity equivalent to those generated by high doses of IL-2 alone.

Human PBL, activated in vitro with IL-2 and treated with hybrid monoclonal antibodies, block growth of human colon carcinoma cells in tumor neutralization assays in nude mice. The hybrid molecules consist of cross-linked monoclonal antibodies, which react 1) with immunoglobulin Fc receptors on K cells or with the antigen receptor complex on cytotoxic T lymphocytes and 2) with human colon carcinoma cells (collaborative effort with Dr. D. Segal, see Project No. Z01-CB-05050-12 I).

Project Description

Objectives: This project has been directed at understanding and manipulating mechanisms of cell-mediated immune cytotoxicity. Long-range goals are: (1) to identify and characterize factors which influence induction, maturation and expression of cytotoxic cellular immune responses particularly against tumor cells; (2) to define changes in cytotoxic cellular immunity which occur in tumor bearing hosts; and (3) to find means of manipulating the cytotoxic cellular immune response for therapeutic purposes.

Methods Employed: Cell-mediated cytotoxicity has been measured in vitro by release of ^{51}Cr from labelled target cells incubated with lymphoid cells for 2-6 hours. Cell-mediated cytotoxicity against syngeneic tumor cells has also been tested in vivo in standard tumor neutralization (Winn) assays in which effector lymphoid cells and tumor target cells are mixed together in vitro at room temperature and within 5 minutes injected subcutaneously into adult hosts. Techniques for human tumor neutralization by human effector cells in nude mice, done in collaboration with Dr. D. Segal, are presented in Annual Report No. Z01-CB-05050-12 I. Cells have been collected from spleen, marrow, lymph node, thymus and peripheral blood. Cells have been fractionated by 1 g velocity sedimentation, density gradient separation in Percoll, removal of cells which ingest iron particles, removal of cells which adhere to anti-Ig coated petri dishes, enrichment of glass adherent cells, removal of cells adherent to nylon or G10 Sephadex, and lysis of cells by monclonal antibodies and complement. Cell purity has been monitored by flow microfluorometry with monoclonal antibodies against well-defined, cell-surface differentiation antigens and by tests of biological function. In addition, effector cells have been produced by in vitro sensitization of lymphoid cells using miniaturized versions of previously established Mishell and Dutton tissue culture techniques. Broadly reactive effector cells have been generated primarily by culturing cells from normal mouse spleen, thymus or fetal liver with polyinosinic acid and/or growth factors. Mouse-derived growth factors have been generated by stimulating EL4 (C57BL leukemia) cells with a phorbol ester. Human-derived growth factor has been used in the form of highly purified recombinant interleukin-2 (IL2) produced in E. coli and obtained from the Cetus Corporation through the courtesy of Dr. S. Rosenberg, NCI. To minimize the occurrance of artifacts related to fetal bovine serum, target cell lines have been adapted to growth in medium supplemented with mouse plasma. This medium also has been used to support cytotoxicity assays. Target cells have been provided by freshly explanted cells (including primary MCA-induced tumor cells dispersed with highly purified collagenase and DNAse), tissue culture lines and established tumor cell lines passed in vivo or in vitro.

Sarcomas have been induced with methylcholanthrene (MCA) in mice which are routinely screened for potentially immunsuppressive pathogens before admission to the colony at the Frederick Cancer Research Center. Tumor cell lines established from these sarcomas are also screened for potentially immunosuppressive pathogens and contaminating virus-related antigens: mycoplasma (culture and serology) and a variety of viruses (serology) including MVM, Sendai, LDH and LCM. The tumor cell lines used in the study have been induced by a relatively low dose of MCA (100ug) and do not stimulate generation

of anti-tumor cytotoxic cells under standard in vitro conditions for generating cytotoxic cells against allogeneic transplantation antigens.

Major Findings: Previous work in this laboratory has demonstrated that normal mouse spleen cells cultured in vitro for 5 days with polyinosinic acid (Poly I) or purified recombinant human IL-2 generate cytotoxic cells whose pattern of target cell reactivity in ^{51}Cr-release assays includes a wide variety of tumor cells but not freshly disbursed or mitogen-stimulated lymphocytes. Even freshly disbursed syngeneic primary tumor cells are lysed by these effector cells, which also block tumor growth in vivo in tumor neutralization (Winn) assays. A close relationship of Poly I-induced anti-tumor cells to natural killer (NK) cells and to lymphokine activated killer (LAK) cells is apparent from a) their development from unprimed lymphoid cells and the absence of stimulatory cells, b) the broad pattern of target cell reactivity, defined by direct lysis and cold target cell inhibition tests, and c) the phenotype of effector cells generated from splenocytes (Thy 1^+, Lyt $1^-,2^-$ -- albeit asialo GM1^-). The response to Poly I is controlled by multiple genes, and there appear to be gene dosage effects (F_1 responses intermediate between high and low parental responses and a unimodal distribution of backcross responses intermediate between the F_1 and low parent responses). With splenocytes from at least two mouse strains showing low responses to Poly I, the IL-2-induced LAK response is suppressed by Poly I. The activity of Poly I is not unique, insofar as other macromolecular polyanions (including carbohydrates) have been identified which have similar activity. The previous work has been extended this year by the following three major findings.

1) The cellular basis for Poly I induction of anti-tumor cytotoxic cells has been partially identified. The response to Poly I requires accessory cells, T cells (primarily L3T4$^+$) and cytotoxic precursor cells. Cytotoxic precursor cells include both Thy 1^- and Thy 1^+ (primarily L3T4$^-$, Lyt 2^-) cells. An essential role of the cells which are not cytotoxic precursors is to produce IL-2, as shown by measurement of IL-2 levels in cultures and by inhibition of IL-2 function with antibodies against IL-2 receptors. Moreover, high levels of pure recombinant IL-2 added to cultures induce generation of cytotoxic cells in the absence of accessory and Thy 1^+ cells. The cytotoxic precursor cell response to low levels of IL-2 is dependent on additional factors (signals), which can be supplied by accessory cells or by Poly I. For example, using Poly I as a second signal, the need for IL-2 to generate a set level of cytotoxic anti-tumor activity can be reduced by at least 90%. Further experiments indicate that splenocytes from different mouse strains differ in their LAK responsiveness to standard doses of exogenous pure IL-2, and thus some of the strain-dependent low responsiveness to Poly I may be due to low responsiveness to endogenous IL-2.

2) The cellular basis for strain-dependent Poly I-induced suppression of Il-2 activated killer (LAK) responses, which involve anti tumor activity, has been partially identified. Suppression requires accessory cells, probably in the macrophage and/or B cell lineages. Suppression is mediated at least in part by prostaglandins in so far as indomethacin partially reverses suppression.

3) Efforts to use cytotoxic anti tumor cells in vivo have been expanded in collaboration with Dr. D. Segal. We have used artificially generated

effector cells consisting of IL-2-activated human PBL treated with anti-tumor hybrid mouse monoclonal antibodies. The hybrid antibodies, developed by Dr. Segal, express binding sites for both tumor cells and effector cells (anti T3 for CTL or anti-immunoglobulin FcR for K cells). The armed human effector cells prevent growth of a human colon carcinoma cell line in tumor neutralization (Winn) assays in nude mice using effector to tumor cell ratios of less then 1:1. The technique has been successfully expanded to a test system for measuring intraperitoneal activity of armed human effector cells against human ovarian carcinoma cells in nude mice, in which host survival is monitored.

Significance to Biomedical Research and the Program of the Institute:

1) In contrast to other forms of lymphocyte-mediated antitumor activity, activated killer cells have shown considerable promise for tumor immunotherapy. Literature reports note that one of the major limitations of current clinical trials involving activated killer cells is the toxicity of associated IL-2 treatment, which has resulted in restriction of IL-2 doses to far less then those needed for optimal anti-tumor effects. The present in vitro studies suggest that the optimal anti-tumor effects of IL-2 can be achieved by using suboptimal doses of IL-2 in conjunction with other factors, such as Poly I. Moreover the present studies suggest a cellular and genetic basis for the failure of some individuals to respond to IL-2 by generation of activated killer cell activity.

2) The tumor neutralization experiments, carried out in collaboration with Dr. Segal, suggest significant clinical applications. Hybrid antibodies which crosslink appropriate sites on human cytotoxic effector cells to human tumor cells augment anti-tumor activity without obvious harmful effects on nude mouse hosts.

Proposed Course of Project: The major objective continues to be identification of the basis for mouse strain-related high versus low activated killer cell responses against tumors.

In collaboration with Dr. Segal, the use of human effector cells armed with hybrid anti-tumor antibodies will be expanded to human ovarian carcinoma and lung tumor immunotherapy models in nude mice.

Publications:

Muller, C. P., Stephany, D. A., and Wunderlich, J. R.: 1986. Changes in mouse MHC expression during the cell cycle of Con A-treated spleen cells. J. Immunol. 136:17-22.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05018-16 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Membrane Damage by Immune Mechanisms

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

PI:	P. A. Henkart	Senior Investigator	IB, NCI
Others:	C. Yue	Medical Staff Fellow	IB, NCI
	W. Munger	Investigator	IB, NCI
	Y. Jiang	Visiting Fellow	IB, NCI
	T. Soares	Microbiologist	IB, NCI
	C. W. Reynolds	Investigator	BTB, FCRF, NCI
	H. Young	Expert	BTB, FCRF, NCI

COOPERATING UNITS *(if any)*

Arnold Greenberg, Manitoba Institute of Cell Biology, Univ of Manitoba, Winnipeg
R. Stevens, Department of Medicine, Harvard Medical School, Boston, MA

LAB/BRANCH

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.)*

The cytoplasmic granules purified from rat large granular lymphocyte tumors with NK activity have been studied to determine their role in cytotoxic and other functions of these lymphocytes. In addition to the major lytic protein termed cytolysin, we have found a series of protease activities present in the purified dense granules. Two of these, which show potent activity against the trypsin thioester substrate known as BLT, have been purified to homogeneity and are among the major granule proteins. One of these enzymes is a disulfide linked dimer of two roughly 30kd protein chains, while the other is comprised of a single chain of about 27kd. These enzymes do not readily hydrolyze protein substrates cleaved by trypsin, but can be shown to act on several synthetic peptide p-nitroanilide substrates with arginine at the P1 site. Inhibitor studies show that both enzymes are serine proteases with a pH optimum of about 8. The pattern of activity inhibition by a panel of inhibitors on the two enzymes is distinct, indicating that these two enzymes do not share a simple monomer-dimer relationship. In addition to these two BLT-hydrolyzing enzymes which have been purified, granule extracts separated by gel filtration show an additional peak of activity against tryptic p-nitroanilide substrates, and 2-3 peaks of activity against chymotryptic p-nitroanilide substrates. Although the physiological roles of these serine proteases are still unclear, each of the two purified BLT-hydrolysing enzymes can synergistically enhance the lysis of nucleated cells by the purified granule cytolysin. Studies of granule proteoglycans have been undertaken by labeling the growing LGL tumors in vivo with ^{35}S-SO_4. It was found that this label is localized in both dense (cytolysin positive) and light (lytically inactive) granule fractions of Percoll fractionated homogenates; kinetic studies suggest that the light granule fraction is a precursor of the dense granule fraction. Western blots of Percoll gradients with rabbit anti-cytolysin antibody show that the cytolysin protein is present in the light granule fraction in spite of the lack of dectable activity.

GPO 914-918

Project Description

Objectives: Our overall objective is to define the mechanisms by which lymphocytes destroy foreign cells. To this end we have dissected the killing process into discrete steps which can be studied independently. In this project we have concentrated on the membrane lesion induced by lymphocytes in the target cell. Over the past few years, we have produced evidence that killer lymphocytes operate by a granule exacytosis mechanism and that one component in these granules is a cytolysin which inserts in the target cell membrane, compromising its permeability properties. Our current objectives are to understand this process at the molecular level and to define the other biologically active molecules in granules of large granular lymphocytes.

Methods Employed: In vivo passaged rat NK tumor cells were harvested from the spleens of leukemic rats, and purified by Ficoll Hypague. These contained $1\text{-}4x10^9$ cells, of which 80-90% are large granular lymphocytes. Cells at 10^8/ml were lysed by nitrogen decompression, the nuclei removed by Nucleopore filtration and 5 ml of the homogerate layered on 20 ml of 48% Percoll. Fractionation of granule components was accomplished by solubilization in 2M NaCl, followed by gel filtration on Ultrogel ACA-54. Cytolysin and the larger BLT esterase were further pruified using a heparin-agarose column, while the smaller BLT esterase was further pruified by affinity chromatography with soybean trypsin inhibitor. Pure granules were harvested from the bottom region of the gradient. Cytolysin activity was measured routinely by serially diluting the material in PBS and adding an equal volume of SRBC suspended in BSS. Hemolysis was measured by hemoglobin release. DNAse was measured by release of ^{125} UDR from nulei. BLT (α-Cbz-lysine-thiobenzyl ester) hydrolysis was measured by a spectrophotometeric assay using Ellman's reagent.

Major Findings: The cytoplasmic granules purified from rat large granular lymphocyte tumors with NK activity have been studied to determine their role in cytotoxic and other functions of these lymphocytes. In addition to the major lytic protein termed cytolysin, we have found a series of protease activities present in the purified dense granules. Two of these, which show potent activity against the trypsin thioester substrate known as BLT, have been purified to homogeneity and are among the major granule proteins. One of these enzymes is a disulfide linked dimer of two roughly 30kd protein chains, while the other is comprised of a single chain of about 27kd. These enzymes do not readily hydrolyze protein substrates cleaved by trypsin, but can be shown to act on several synthetic peptide p-nitroanilide substrates with arginine at the P1 site. Inhibitor studies show that both enzymes are serine proteases with a pH optimum of about 8. The pattern of activity inhibition by a panel of inhibitors on the two enzymes is distinct, indicating that these two enzymes do not share a simple monomer-dimer relationship. In addition to these two BLT-hydrolyzing enzymes which have been purified, granule extracts separated by gel filtration show an additional peak of activity against tryptic p-nitroanilide substrates, and 2-3 peaks of activity against chymotryptic p-nitroanilide substrates. Although the physiological roles of these serine proteases are still unclear, each of the two purified BLT-hydrolysing enzymes can synergistically enhance the lysis of nucleated cells by the purified granule cytolysin. Studies of granule proteoglycans have been undertaken by labeling the growing LGL tumors in vivo with $^{35}S\text{-}SO_4$. It was found that

this label is localized in both dense (cytolysin positive) and light (lytically inactive) granule fractions of Percoll fractionated homogenates; kinetic studies suggest that the light granule fraction is a precursor of the dense granule fraction. Western blots of Percoll gradients with rabbit anti-cytolysin antibody show that the cytolysin protein is present in the light granule fraction in spite of the lack of dectable activity.

Significance to Biomedical Research and the Program of the Institute: The process of lymphocyte destruction of foreign cells may be one of the most important mechanisms for the immunological rejection of allografts and tumors in vivo. Studies such as ours, directed at the cellular and molecular nature of this process, allow a more complete understanding of the basic knowledge of the body's immunological defense system against foreign cells, including malignant cells.

Proposed Course of Project: Efforts to obtain an amino terminal amino acid sequence of the cytolysin protein continue in the expectation that this will lead to the identification of the gene coding for this protein from the cDNA library produced from the LGL tumor cells by Dr. Howard Young. The complete sequence of a gene selectively expressed in CTL, LGL, and helper T cells has been elucidated by Gershenfeld and Weissmann, of Stanford and was shown to code for a serine protease with tryptic specificity. We are currently collaborating with Dr. Lee Maloy who has produced synthetic peptides corresponding to predicted exposed protein loops of this enzyme. Anti-peptide antibodies are being raised in order to see if one of the three granule tryptic enzymes correspond to this gene. Purification of the remaining granule proteases has begun, and will proceed to completion if protein levels are adequate. Various protein substrates for these enzymes are being tested in order to ascertain their physiological function. The mechanism of the synergism of cytolysin and BTL esterases in lytic functions is being examined.

Publications:

Henkart, P.: The granule exocytolsi mechanism for lymphocyte cytotoxicity In: Mechanisms Of Host Resistance To Infectious Agents, Tumors, and Allografts (Steinman, R. M., North, R. J., Eds.). Rockefeller University Press, New York, 1986, pp. 205-216.

Henkart, P., Henkart, M., Millard, P., Fredrikse, P., Bluestone, J., Blumenthal, R., Yue, C., Reynolds, C. W.: 1985. The role of cytoplasmic granules in cytotoxicity by large granular lymphocytes and cytotoxic T lymphocytes. Adv. Exp. Med. Biol. 184:121-134.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05021-15 I

PERIOD COVERED: October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Antigens Determined by the Murine Major Histocompatibility Locus

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

PI:	D. H. Sachs	Chief, Transplantation Biology Section	IB, NCI
Others:	J. A. Bluestone	Laboratory Leader	IB, NCI
	S. Chatterjee-Das	Guest Researcher	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 20205

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.)*

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; and 5) Mixed allogeneic and xenogeneic chimeras, in which irradiated animals are reconstituted with mixtures of T-cell depleted donor and host marrow, are produced and the mechanism of tolerance and of immune responsiveness in these animals is studied in vivo and in vitro.

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

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. One cross-reactive specificity of an anti-H-2K^b antibody (20-8-4) has been shown to define a Qa antigen determined by gene(s) to the right of the MHC. This finding indicates that cross-reactions between class I products may be useful in detecting differentiation antigens encoded by the numerous class I genes now known to be present in the genome.

2) Analysis of anti-H-2 antisera and monoclonal antibodies by gel electrophoresis of labeled cell surface antigens has demonstrated two new H-2 products determined by loci within the D region of H-2^q, named H-2L and H-2R. A large panel of monoclonal antibodies directed to products of H-$2D^q$ have been produced and characterized, and have been used for immunoprecipitation analyses. These studies have demonstrated the existence of at least one additional class I antigen determined by the H-2D region.

3) 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 a large number of monoclonal antibodies directed to T cell receptors against MHC antigens is presently being developed.

4) 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 non-specific suppressor cell generation 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 radiation sensitive, and may be involved in the first phase of elimination of alloreactive cells.

5) Assessment of genetic specificity of transplantation tolerance in mixed allogeneic chimeras has indicated that bone marrow reconstitution tolerizes completely to MHC antigens, but incompletely to skin-specific minor antigens encoded outside of the MHC. Similar skin-specific antigens may explain partial tolerance in the mixed xenogeneic (mouse plus rat) chimeras as well. Analysis of the genetics in this system has been carried out by grafting skin from backcross animals (B10.BRxC3H)F_1 backcrossed to B10.BR to mixed C3H+B10->C3H

chimeras which had rejected a C3H skin graft. Preliminary results indicate that one strong and one or two weaker antigen systems are involved.

6) 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.

Publications:

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Sachs, D. H., Bluestone, J. A., and Epstein, S. L.: Idiotypes of anti-MHC receptors. In Streilein, J. W., Ahmad, F., Black, S., Blomberg, B., and Voellmy, R. W. (Eds.): Advances in gene technology: Molecular biology of the immune system. ICSU Short Reports Vol. 2, Cambridge University Press, 1985, pp. 101-104.

Ildstad, S. T., Wren, S. M., Bluestone, J. A., Barbieri, S. A., Stephany, D., and Sachs, D. H.: Effect of selective T cell depletion of host and/or donor bone marrow on lymphopoietic repopulations, tolerance, and graftversus-host disease in mixed allogeneic chimeras (B10+B10.D2=>B10). J. Immunol. 136, 28-33, 1986.

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Maloy, W. L., Ozato, K., Sachs, D. H., and Coligan, J. E.: Evidence for the association of I-A and I-E molecules in d-haplotype mice. Mol. Immunol. 23: 263-269, 1986.

Chatterjee-Das, S., Schlauder, G. G., Sachs, D. H., Glimcher, L. H., Paul, W. E., and McKean, D. J.: A biochemical analysis of I-A^k molecules from mutant antigen presenting cell lines. Immunogenetics 23: 121-125, 1986.

Ildstad, S. T., Bluestone, J. A., and Sachs, D. H.: Alloresistance to engraftment of allogeneic donor bone marrow is mediated by an $LYT2^+$ T cell in mixed allogeneic reconstitution (C57BL/10Sn + B10.Ds/Sn => C57BL/10Sn). J. Exp. Med. 163: 1343-1348 1986.

Simpson, E., Lieberman, R., Ando, I., Sachs, D. H., Paul, W. E., and Berzofsky, J. A.: How many class II immune response genes: A reappraisal of the evidence. Immunogenetics, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05023-15 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

PI:	D. H. Sachs	Chief, Transplantation Biology Section	IB, NCI
Others:	S. A. Rosenberg	Chief, Surgery Branch	SB, NCI
	E. O. Kortz	Medical Staff Fellow	IB, NCI
	R. E. Gress	Medical Officer	IB, NCI
	K. Pratt	Biotechnology Training Program Fellow	IB, NCI
	D. S. Singer	Senior Investigator	IB, NCI
	K. Sakamoto	Visiting Fellow	IB, NCI

COOPERATING UNITS *(if any)*
NIH Animal Center, Poolesville, Maryland
J. K. Lunney, Research Chemist, USDA Animal Parasitology Institute, Beltsville, MD

LAB/BRANCH
Immunology Branch

SECTION
Transplantation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

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: two intra-MHC recombinants have been obtained and bred to homozygosity. Kidney transplants utilizing these new recombinants have shown that selective matching for Class II antigens frequently permits long-term kidney graft survival across a Class I difference. An additional MHC recombinant has been obtained recently which appears to provide a new combination of class I and class II genes; 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 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 in the EMBL-3 phage vector has been constructed and screened with these probes. Class I and class II genes from the pig herds are being characterized.

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

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 three lines bearing intra-MHC recombinants.

2) Tranplants 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 their 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.

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, and show extensive cross-hybridization between β chains of the three major loci.

6) Complete cosmid and phage libraries have been constructed from the d and c haplotypes, and several class I and class II genes have been isolated and characterized.

7) A series of monoclonal antibodies reactive with a variety of swine lymphocyte surface antigens have been prepared. One of these antibodies recognizes an SLA antigen of the SLA^{dd} haplotype, at least two others react with selective T cell subpopulations, and at least one antibody is reactive with macrophages. Further characterization of these antibody reactivities is in progress.

Publications:

Pescovitz, M. D., Popitz, F., Sachs, D. H., and Lunney, J. K.: Expression of Ia antigens on resting porcine T cells: A marker of functional T cell subsets. In Streilein, J. W., Ahmad, F., Black, S., Blomberg, B., and Voellmy, R. W. (Eds.): Advances in gene technology: Molecular biology of the immune system. ICSU Short Reports Vol. 2, Cambridge University Press, 1985, pp. 271-272.

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Lunney, J. K., Pescovitz, M. D., and Sachs, D. H.: The swine major histocompatibility complex: Its structure and function. In Tumbleson, M. E. (Ed.): Swine in Biomedical Research. Plenum Press, New York, in press.

Sakamoto, K., Pennington, L. R., Popitz-Bergez, F. A., Pescovitz, M. D., Gress, R. E., McDonough, M. A., Shimada, S., Katz, S. I., and Sachs, D. H.: Swine GVHD model and the effect of T cell depletion of marrow by monoclonal antibodies. J. Cell. Biochem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05033-15 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Immunotherapy of Human Cancer

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

PI:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
Others:	S. A. Rosenberg	Chief	SB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

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.

Project Description

Objectives: This project is designed to study various approaches to the immunotherapy of human malignancies.

Methods Employed: A new protocol (designed IB-2) was initiated in August 1975. This protocol evaluated the effect of two types of immunotherapy or one type of chemotherapy on the remission duration and survival of patients with stage I (level 4 or 5) and stage II malignant melanoma. Patients were stratified by stage, site of primary, and for stage II patients, presence or absence of clinically palpable lymph nodes and the number of histologically positive lymph nodes. Following stratification, they were randomized to receive no further therapy (this is standard treatment for these patients); or chemotherapy with methyl-CCNU; or immunotherapy with BCG; or immunotherapy with BCG plus a vaccine that consists of three tissue culture-grown allogeneic melanoma cell lines treated with the enzyme neuraminidase to remove sialic acid (and thus render them more immunogenic) and frozen until ready for use.

Following randomization, patients were assigned to the Medicine Branch for chemotherapy, the Immunology Branch for immunotherapy, and the Surgery Branch for follow up if there is no further treatment.

Bloods were drawn during pre-randomization work-up and throughout the treatment cycle for the purpose of serum banking.

Major Findings: As of Dec. 1, 1982, 181 patients had been randomized into this protocol. The state of the trial is summarized in the following table:

	Control	MeCCNU	BCG	BCG & Vaccine
Total Patients Entered	43	46	47	45
Recurrences	32	25	38	25
Deaths	24	23	27	21

In vitro assays have only been carried to the point of indicating that patients are being effectively immunized with the vaccine (see Project No. Z01-CB-05016-10 I). In addition, PPD tests indicate that all patients receiving BCG have converted to a positive skin test.

Significance to Biomedical Research and the Program of the Institute: Immunotherapy studies will explore the clinical effectiveness of manipulating the immune system in patients with cancer, and will also provide new information about the biology of the tumor-host relationship.

Proposed Course of Project: No further patient accrual will occur. All treatment has been completed and, for those patients already on study, follow-up as described by the IB-2 protocol will be continued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05035-14 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

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	IB, NCI
Others:	F. Uher	Postdoctoral Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	0.75	1.00

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

The goal of this project is to characterize the function of B lymphocyte 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 and monoclonal anti-Fcγ receptor antibodies indicate that B lymphocyte Fcγ receptors cross-linked by their physiologic ligand down-regulate B lymphocyte differentiation without affecting proliferation. Resting but not activated B lymphocytes are susceptible to this negative regulation. Occupancy of B lymphocyte surface IgM by a separate ligand is necessary for inhibition to occur, suggesting that the previously described interaction between these two membrane receptors may be involved in generating the negative signal. Monoclonal anti-Fcγ receptor antibodies on a Sepharose matrix but not in soluble form affect B lymphocyte function in a fashion similar to antigen antibody complexes. This result suggests that certain monoclonal antibodies specific for cell surface receptors must be presented in a form which will produce effective cross-linking in order to obtain functional effects. Various B lymphocyte populations have been evaluated for susceptibility to Fcγ receptor mediated downregulation. 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.

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

Project Description

Objectives: Characterization of the function of B lymphocyte Fcγ receptors in regulation of the responses of B lymphocytes.

Methods Employed: B lymphocytes are prepared from spleens of mice by rigorous depletion of T lymphocytes and in some cases depletion of antigen-presenting cells. The B lymphocytes are cultured with various types of antigen-antibody complexes and/or monoclonal anti Fcγ receptor antibody (as a model ligand) in various forms. Cultures with ligand alone are assessed for stimulation of responses, while cultures containing both ligand and a) $F(ab')_2$ anti-mu, b) lymphokines, c) both $F(ab')_2$ anti-mu and lymphokines, and d) the mitogen LPS (as stimulatory signals) are assessed for proliferation (^{3}H-thymidine incorporation) and antibody secretion (either by direct or reverse plaque forming cell assay or Elisa analysis of culture supernatants). Variables assessed include the characteristics of the ligand (valence, concentration, etc.), kinetics of signal generation, type of B lymphocyte (subpopulations, state of activation, immunodeficient or autoimmune B cells, etc.) and requirement for other molecules in signal generation.

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 indicated that in soluble form they did not affect B lymphocyte responses. However, when on a Sepharose matrix they specifically inhibited B lymphocyte antibody production but had little effect on proliferation. Thus, provided that sufficient cross-linking can be obtained, the specific monoclonal antibody has effects similar to the specific ligand when bound to B lymphocyte Fcγ receptors. Chemically cross-linked monoclonal anti-Fcγ receptor antibodies have been prepared by a variety of protocols in an attempt to obtain soluble multimers which would generate the downregulatory signal and which could be used in vivo. However, none of the preparations to date has generated the downregulatory signal. Experiments are in progress to determine the extent that these multimers cross-link Fcγ receptors.

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/l 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.

Significance to Biomedical Research and the Program of the Institute: Therapeutic manipulation of humoral immune responses both in cancer patients and other diseases ultimately depends on an understanding of the B lymphocyte membrane molecules which function as receptors for immunoregulatory signals. Fcγ receptors are unique in that they are the only B lymphocyte membrane molecule so far identified which upon interaction with their specific ligand downregulate B lymphocyte responses. Thus, Fcγ receptors may offer a new therapeutic approach to abbrogating pathogenic antibody responses such as in autoimmune disease. Moreover, it is possible that Fcγ receptor mediated downregulation plays a role in B lymphocyte self-tolerance.

Proposed Course of Project: 1) Covalently cross-link monoclonal anti-Fcγ receptor antibody to various other antibodies specific for B lymphocyte membrane molecules in order to produce soluble multimers which will generate Fcγ receptor mediated downregulation for use in vivo. 2) Evaluate the effects of Fcγ receptor blockade using native monoclonal anti-Fcγ receptor antibody on generation of B cell tolerance. 3) Evaluate Fcγ receptor expression following exposure of B cells to various stimuli. 4) Evaluate the role of Fcγ receptors in lymphocyte resistance or susceptibility to viral infection. 5) Evaluate the role of Fcγ receptors in B lymphocyte antigen presentation to T lymphocytes.

Publications:

Uher, F., Lamers, M. C., and Dickler, H. B.: Antigen-antibody complexes bound to B-lymphocyte Fcγ receptors regulate B-lymphocyte differentiation. Cellular Immunol. 95:368-379, 1985.

Uher, F. and Dickler, H. B.: Independent ligand occupancy and cross-linking of surface Ig and Fcγ receptors downregulates B lymphocyte function. Evalulation in various B lymphocyte populations. Molecular Immunol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05036-14 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic Control of the Immune Response to Staphylococcal Nuclease

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:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
	A. Finnegan	Guest Worker, Immunotherapy Section	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION
Transplantation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Several hybridomas reactive with nuclease and/or anti-idiotype have been produced and characterized. Syngeneic anti-idiotypes have also been produced and are presently being characterized in both antibody and T cell systems. Competitive binding studies are used to determine epitopes of nuclease as defined by available monoclonal antibodies. Examination of the kinetics of inhibition of nuclease with combinations of monoclonal antibodies is being used to determine mechanism of inhibition of catalytic activity of this enzyme. Site-directed mutagenesis of the nuclease gene has provided numerous point mutants of nuclease which are being studied for changes in immune reactivity. Synthetic peptides of nuclease are being examined to determine the relative importance of specific sites for interacting with T cells and antibodies.

Major Findings: Several hybridomas producing anti-nuclease antibodies have been prepared from immunized BALB/c, A/J and SJL mice immunized with nuclease. Also, several hybridomas producing idiotype without detectable antigen binding activity (ID') have been prepared from BALB/c mice treated with anti-idiotype.

Monoclonal antibodies to nuclease have been divided into at least three groups according to their epitope binding, as assessed by competitive inhibition of ELISA. Additional monoclonal antibodies have been produced and assessment of their epitope binding is in progress.

A series of mutants of nuclease have been prepared by site-specific mutagenesis in the laboratory of Dr. David Shortle, and have been made available to us for studies of antibody and T cell binding. So far, all of the mutants appear to react normally with our monoclonal antibody populations. However, several differences have been detected in panel tests of cloned proliferative T cells.

Publications:

Devaux, C. A., El-Gamil, M., and Sachs, D. H.: Binding of anti-nuclease monoclonal antibodies to functionally defective Staphylococcal nuclease variant proteins obtained by site-directed mutagenesis of the NUC gene. In Streilein, J. W., Ahmad, F., Black, S., Blomberg, B., and Voellmy, R. W. (Eds.): Advances in gene technology: Molecular biology of the immune system. ICSU Short Reports Vol. 2, Cambridge University Press, 1985, pp. 139-140.

Devaux, C. A., Nadler, P. I., Miller, G. G., and Sachs, D. H.: Genetic control of immune response to Staphylococcal nuclease. XII. Analysis of nuclease antigenic determinants using anti-nuclease monoclonal antibodies. Mol. Cell. Biochem. 68: 31-40, 1985.

Devaux, C. A., Pierres, M., Epstein, S. L., and Sachs, D. H.: Xenogeneic antibodies with apparent public idiotypic specificity for anti-Ia.7 antibodies are directed in part against V_k21D and E subgroup marker. J. Immunol. 136: 3760-3766, 1986.

Devaux, C. A., Nadler, P. I., El-Gamil, M., Miller, G. G., and Sachs, D. H.: Genetic control of immune response to Staphylococcal nuclease. XII. Analysis of nuclease antigenic determinants using anti-nuclease monoclonal antibodies. Molec. Cell. Biochem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05050-12 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Studies of Immunologically Relevant Cell Surface Phenomena

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

PI:	D. M. Segal	Senior Investigator	IB, NCI
Others:	D. P. Snider	Visiting Fellow	IB, NCI
	J. Wunderlich	Senior Investigator	IB, NCI
	T. Hecht	Associate Professor	Univ. MD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 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.)*

1. Cloned cytotoxic T cells and fresh peripheral blood T cells have been targeted against human tumor cells by coating them with anti-T3 cross linked to anti-tumor cell monoclonal antibodies. Such retargeted T cells lyse cultured human tumor lines with the same specificity as the monoclonal anti tumor antibody. When tested on fresh normal or neoplastic tissue, retargeted human peripheral blood T cells selectively lysed the fresh neoplastic cells.

2. Peripheral blood K cells have been retargeted using anti-FcR cross linked to anti-target cell antibodies. The effector cells are Leu 11^+, $T3^-$, and approximately 30% $T8^+$. These same cells also mediate NK activity in vitro, especially when stimulated with IL-2. The cytotoxic cells which are targeted with anti-FcR cross linked to anti-target cell antibodies are clearly different from, but have about the same lytic activity as the cells which are targeted with anti-T3 cross linked to anti target cell antibodies.

3. Both retargeted human T and retargeted human K cells show potent anti-tumor activity in Winn type tumor neutralization assays in nude mice. In these studies, the growth of LS174T, a human colon carcinoma was totally blocked at effector:target ratios less than 1:1.

Project Description

Objectives: (1) To investigate molecular and cellular interactions which occur during the generation and effector phases of cellular and humoral immune responses, and (2) to find methods by which these interactions can be artifically manipulated with the ultimate goal of increasing immune responses against neoplastic or otherwise pathogenic cells and decreasing autoimmune responses.

Methods Employed: Gel filtration, protein cross linking, ion exchange chromatography, polyacrylamide gel electrophoresis, complement fixation, radiolabeling of proteins, tissue culture, binding assays, cell and complement-mediated cytotoxicity assays, proliferation assays, cell separations, flow microfluorometry, computer analyses.

Major Findings:

1. Retargeting of Peripheral Blood T Cells Against Human Tumor Cells: We and others have previously shown that cytotoxic T cells (CTL) can be induced to lyse cells they normally would not lyse by coating them with anti-T cell receptor antibodies which have been covalently cross linked to anti-target cell antibodies. In particular, the $T8^+$ subset of fresh human peripheral blood T cells can be targeted using anti-T3 cross linked to anti-target cell antibodies, and the activity of these cells is enhanced by brief (4 hr) exposure to human recombinant IL-2.

Earlier studies were done using convenient laboratory antigens such as TNP, MHC class I antigens, and Thy. We asked whether we could also use this technique to specifically lyse tumor cells, using anti-T3 cross linked to anti-tumor cell antibodies. Several anti-human tumor monoclonal antibodies were obtained from a variety of sources. Many of these have been tested on normal tissue and have been shown to be relatively tumor specific. Some of the anti-tumor antibodies which we have used are; 315F6, raised against a human non-small cell lung cancer line; 8H12, raised against a human breast cancer line; 96.5, raised against a human melanoma line, and HeFi-1, raised against a Reed-Steinberg line. These antibodies were tested for binding on a panel of human tumor lines by fluorescence flow cytometry, and were found to vary in their cross reactivities. For example, 315F6 cross reacted on all tumor lines tested, including B lymphoblastoid, Reed-Steinberg, small and large cell lung cancer, and colon lines. 8H12 was somewhat more specific, cross reacting only on small cell lung and colon cancer lines. HeFi-1 bound only to the Reed-Steinberg line. When cross linked to anti-T3, each of these antibodies caused human peripheral blood T-cell to lyse the cultured tumor lines with the same specificity as determined from the fluorescent binding assays.

Next, we tested the targeted T cells for the ability to lyse fresh normal or neoplastic cells, in vitro. Fresh, human T cells, when treated with the appropriate cross linked antibody lysed fresh human tumors with varying lytic activities and specificities. For example, anti-T3 x 96.5 (anti-T3 cross lined to the antimelanoma antibody, 96.5) caused peripheral blood T cells to lyse fresh human melanoma cells, but not fresh normal liver, lung, kidney, or neoplastic colon or lung cells. Anti-T3 x 8H12 resulted in the lysis of fresh neoplastic colon cells from 2 patients but not normal liver,

lung, kidney or neoplastic lung or melanoma. However this cross linked antibody preparation caused normal colon cells to be lysed to a small degree (when compared to lysis of neoplastic colon). Interestingly anti-T3 x 315F6, the cross-link which promoted lysis of most cultured tumor lines, showed specificity only for neoplastic melanoma, when tested against fresh normal and tumor tissue.

Thus, the cross linked antibodies are able to specifically target human peripheral blood T cells against fresh human tumor cells in vitro. Based upon these results, it is reasonable to test this system in vivo (first in experimental animals, and then in cancer patients) as a novel form of cancer immunotherapy.

2. Retargeting of Human Peripheral Blood K Cells With Cross Linked Antibodies. We originally showed that anti-Fc receptor cross linked to anti-target cell antibody would cause murine antibody dependent cell mediated cytolytic (ADCC) effector cells to lyse specified target cells. More recently we have used this same strategy to retarget human peripheral blood K cells against various target cells. The hetero-cross linked antibodies used in these studies contained 3G8, which binds to an epitope on the K cell Fc receptor (FcR).

Fresh human peripheral blood lymphocytes, when treated with anti-FcR x anti-target show strong lytic activity against designated target cells, including cultured murine and human tumor cells and chicken red blood cells. The effector cells mediating this lysis are clearly different from those mediating lysis in the presence of anti-T3 x anti-target as judged by depletion studies. For example, anti-Leu 11b + complement totally abrogates the anti-FcR x anti-target activity but not that mediated by anti-T3 x anti-target. On the other hand, anti-T8 + complement totally eliminates the T3-dependent lysis but only partially eliminates the FcR-dependent lysis. In short, cells mediating lysis in the presence of 3G8 x anti-target are $T3^-$, Leu 11^+ and approximately 30% $T8^+$. Those mediating lysis in the presence of anti-T3 x anti-target are $T3^+$, Leu 11^- and 100% $T8^+$.

The K cells largely, if not totally, overlap the NK population. In the presence of IL-2, these cells become highly lytic, in vitro; for most types of tumor cells in the absence of antibody (i.e., NK activity). In fact we have never seen activation of the antibody dependent component by IL-2, in the absence of activation of the antibody-independent component. Therefore, it is not possible to say, from in vitro results, whether the 3G8 x anti-target activity is activated by IL-2.

3. Tumor Neutralization Assays: In collaboration with Dr. John Wunderlich, we have tested the ability of human effector cells coated with cross linked antibodies to prevent tumor growth subcutaneously in nude mice. LS174T, a human colon cancer line grows subcutaneously in nude mice, giving very large tumors in about 30 days. This tumor also expresses antigens recognized by 315F6 and 8H12, and is killed in vitro, by human peripheral blood lymphocytes coated with anti-T3 x 315F6, anti-T3 x 8H12, anti-FcR x 315F6 and anti-FcR x 8H12. We injected LS174T subcutaneously in the presence or absence of fresh peripheral blood lymphocytes coated with various combinations of these antibodies, or without antibody. In most studies the Fab

fragments were cross linked, rather than the intact antibodies, to eliminate the possibility that normal ADCC was contributing to the results.

Our studies have shown that (1) there is potent tumor neutralization activity by fresh human PBL coated with cross linked antibodies containing either anti-T3 or anti-FcR. For example, in one experiment anti-T3 x 315F6 coated PBL totally prevented tumor growth (in 5 mice) at an effector target (E:T) ratio of 2:1, and partially protected at an E:T of 0.7:1. Since only about 1/3 of the PBL could be effector cells (i.e., are $T8^+$), good protection is seen at effective E:T ratios well below 1. Even better activity was seen using the anti-FcR cross link. Here complete protection was observed at an E:T of 0.4:1, in a system where only 10% of the PBL could have been effector cells (i.e., FcR^+). Cross linked antibody on the effector cells was required for lysis; no protection was observed in its absence.

Interesting, a great enhancement in activity was not observed when effectors were pretreated with IL-2, using T cells as effectors. By contrast a substantial increase was seen when the K cells were the effectors. In one experiment, a substantial increase in activity was seen when both anti-T3 x 8H12 and anti-T3 x 315F6 were used together, compared with either alone. In another experiment, however, not much difference was seen. These studies demonstrate that effector cells coated with cross linked antibodies can prevent tumor growth in an in vivo environment. However since effectors and targets were administered together, these experiments do not indicate whether antibody-coated effector cells could kill an established tumor if they were, for example, injected into a cancer patient.

4. Ovarian Cancer Models: We are currently setting up an ovarian cancer model in nude mice to see if targeted effector cells can prevent growth of an established tumor. We are using OVCAR3, a human ovarian cancer line which grows interperitoneally in nude mice in much the same way that ovarian tumors grow in humans. We have cross linked two anti-ovanian monoclonal antibodies to anti-FcR and to anti-T3. The antibodies were raised by Dr. Toby Hecht of the University of Maryland, who is a collaborator on this project, and the cross linked preparation cause human PBL to lyse OVCAR3 in vitro.

We have confirmed that OVCAR3, when injected I.P. into nude mice in the proper dosage, will grow and kill the mice in 4-6 weeks. In our first test, tumor bearing mice were given human PBL I.P. at various times after receiving the tumor. Preliminary results suggest that if multiple injections of effector cells are given, tumor growth can be prevented. In this experiment tumor did grow in mice given effectors without antibody but did not grow when antibody coated effectors were given 2 or 3 times. Reproducibility in this system has not yet been established.

Significance to Biomedical Research and the Program of the Institute: The recognition of foreign substances by antibodies elicits a number of reactions which normally leads to their elimination from the body. The purpose of this project is to examine the molecular events which occur as a result of antigenic recognition. It is hoped that these studies will enhance our understanding of these processes and allow us to better control immune reactions. An immediate goal is to enhance immune responses toward neoplastic cells.

Proposed Course of Project:

1. We will continue studies with the ovarian system in order to find the best way of preventing tumor growth. We plan to vary injection times, numbers of doses of effector cells and sizes of dose. IL-2 will be injected IP in tumor bearing mice which have received antibody coated effector cells to see if the tumoricidal activity of the effector cells can be enhanced. Tumor destruction will also be followed by measuring the levels of CA125 antigen in ascites and in blood.

2. Depending upon the in vivo results with nude mice, we plan to initiate a clinical protocal to investigate whether antibody coated effector cells can be used therapeutically in patients suffering from ovarian cancer.

3. We will next extend these studies to other cancers, for example, lung.

4. We have initiated experiments for the purpose of using cross linked antibodies to manipulate other aspects of the immune system, for example, helper T-cell function and antigen presentation.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05058-11 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Immunoregulation by Anti-idiotype Antibodies

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

PI:	H. B. Dickler	Senior Investigator	IB, NCI
Others:	G. Berrebi	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

Dr. Seth Pincus, University of Utah

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The goal of this project is to characterize the mechanisms by which anti-idiotype antibodies regulate immune responses and lymphocyte function. A system has been developed in which, for the first time, soluble antibody responses to the synthetic polypeptide (T,G)-A--L can be generated and detected in vitro using antigen-primed lymph node cells. Responses are antigen dependent and specific, and H-2 linked Ir gene regulated. Antibodies specific for the idiotypes of anti-(T,G)-A--L antibodies induce antigen-independent anti-(T,G)-A--L antibody responses. These responses are specific at the levels of the anti-idiotype reagent, the antigen-priming, and the antibody produced. The anti-idiotype antibodies stimulate function from antigen-primed T lymphocytes in the form of soluble helper lymphokines, and function from both primed and unprimed B cells in the form of specific antibody secretion. Unprimed B cells, in addition to anti-idiotype, require either primed T cells or idiotype or unrelated antibody complexes to be present in order to obtain function. Responses to anti-idiotype antibodies, in contrast to those to antigen, appear not to be regulated by Ir genes. A monoclonal anti-idiotype which reacts with a public idiotope present on the majority of anti-(T,G)-A--L antibodies from mice of the IgCH b haplotype e.g., B10 but absent on such antibodies from mice of the IgCH a haplotype e.g., Balb.B has been obtained. Expression of this idiotype has been assessed in anti-(T,G)-A--L responses utilizing T and B lymphocyte mixtures prepared with cells from idiotype positive and negative strains in all combinations. No evidence was obtained for an idiotype-regulating T helper lymphocyte. The monoclonal anti-idiotype in soluble form failed to stimulate anti-(T,G)-A--L responses in vitro.

Project Description

Objectives: Characterization of the mechanisms by which anti-idiotype antibodies regulate immune responses and lymphocyte function.

Methods Employed: An in vitro microculture system using antigen-primed lymph node cells is utilized. Antibody responses are measured using an enzyme-linked immunoabsorbant assay (Elisa) with antigen-coated plastic plates. Immunoregulation is studied by manipulation of the conditions of interaction, and by immunologic and chemical modifications of both the lymphocytes and reagents. Anti-idiotype antibody is prepared by immunization of Lewis rats with antigen-affinity purified murine anti-(T,G)-A--L antibodies with subsequent adsorption on normal mouse immunoglobulins. Anti-idiotype activity can be measured by inhibition of binding of anti-(T,G)-A--L antibodies to the antigen, or by direct binding to the idiotype. Genetic studies are carried out utilizing inbred, congenic, and/or recombinant mouse strains. Lymphocyte subpopulations are prepared by a variety of techniques including antibody and complement mediated lysis, antibody affinity plates, density gradients, and adherence columns. Other techniques employed include flow microfluorometry and production and purification of monoclonal antibodies. A monoclonal anti-(T,G)-A--L anti-idiotype antibody was prepared by standard hybridoma technology using immune Lewis rat spleen cells from a Lewis rat immunized as described above.

Major Findings: Using a microculture system we have been able to obtain antigen-specific, T lymphocyte dependent responses to (T,G)-A--L in vitro. This is the first known success at obtaining a soluble antibody response in vitro to an Ir gene regulated antigen. The characteristics of the system are as follows: 1) In order to detect such responses it is necessary to wash the antigen out of the cultures after 3 days and use a very sensitive detection system (Elisa); 2) Primed T lymphocytes are required; 3) The response is antigen-dependent and specific; 4) The response is under Ir gene control and the Ir genes are phenotypically expressed by B lymphocytes and/or accessory cells; 5) T and B lymphocytes can cooperate to produce these responses if they are H-2 identical even if non-H-2 genes are different; 6) Responses are highly reproducible and levels of antibody are 100-500 ng/ml.

We have evaluated the effects of anti-idiotype reagents in this system. It was found that antigen-primed lymph node cells responded to anti-idiotype by the production of antibody in the absence of antigen. This antigen-independant response was specific at the level of the anti-idiotype reagent in that other reagents including normal rat serum, rat anti-mouse IgG, rat anti-mouse IgM and rat anti-nuclease anti-idiotype were not active. Moreover, idiotype affinity-purified anti-idiotype was active. The response was also specific at the level of priming in that cells primed to CFA or ovalbumin would not respond to anti-(T,G)-A--L anti-idiotype. Finally, it was specific at the level of the antibody produced since antibodies against other antigens were not induced.

The cellular site of action of the antigen-independent anti-idiotype induced anti-(T,G)-A--L response was evaluated. Rigorously B lymphocyte depleted antigen-primed T lymphocytes together with equal numbers of unprimed B lymphocytes (unprimed lymphocytes, B or T, would not respond to anti-idiotype)

responded to anti-idiotype, suggesting that the anti-idiotype was provoking function from T helper lymphocytes. This was directly shown by stimulating antigen-primed T cells for 3 days with anti-idiotype and collecting the supernatant. This supernatant (but not controls) contained soluble helper lymphokines which allowed unprimed B cells to produce specific antibody when stimulated with anti-idiotype (but not controls). Thus, the anti-idiotype directly triggers both T and B lymphocytes. The T lymphocytes which respond to anti-idiotype have been characterized as Lyt 1 positive, Lyt 2 negative. Responses to the anti-idiotype do not appear to be regulated by Ir genes. Thus, mixtures of (Responder x Nonresponder)F_1 antigen-primed T lymphocytes with Nonresponder B plus accessory cells respond to anti-idiotype but not to the antigen.

Antigen-primed B lymphocytes rigorously depleted of T cells specifically respond to anti-idiotype by secretion of anti-(T,G)-A--L antibodies. This response was shown to be specific by a variety of criteria. This stimulation of function by anti-idiotype appears to involve idiotype-anti-idiotype complexes because normal B cells respond to neither idiotype or anti-idiotype but do respond to a mixture of the two. Moreover, Fcγ receptors appeared to be involved in that normal B lymphocytes would also respond in the presence of anti-idiotype plus unrelated antibody complexes.

A rat γ2b,k monoclonal antibody produced by a hybridoma (I-9) has been obtained in collaboration with Dr. Seth Pincus, University of Utah. This antibody reacts with a public idiotope present on the majority of anti-(T,G)-A--L antibodies from mice of the IgCH b haplotype but not on such antibodies from mice of the IgCH a haplotype. The idiotype appears to be near the combining site. In soluble form this monoclonal anti-idiotype has failed to stimulate anti-(T,G)-A--L responses in vitro.

Anti-(T,G)-A--L responses were obtained in vitro from mixtures of T and B lymphocytes prepared in all combinations of cells from idiotype positive and negative strains. Idiotype expression was not affected by the IgCH haplotype of the T helper lymphocytes. Thus, no evidence was obtained for the existance of an idiotype regulating T helper lymphoycte.

In order to evaluate supernatants from T lymphocytes stimulated by anti-idiotype for helper function a new assay has been developed. Unprimed B lymphocytes are incubated with primed helper T lymphocytes and antigen for 48 hrs. The T lymphocytes and antigen are then eliminated and antigen-specific responses are obtained in the presence but not the absence of helper lymphokines. This assay will allow evaluation of the nature of helper lymphokines generated by the interaction of anti-idiotype and helper T lymphocytes.

Significance to Biomedical Research and the Program of the Institute: An understanding of the regulation of the immune response could lead to new forms of therapy for human diseases including cancer. Specifically, the findings outlined here indicate it is possible to trigger antigen-specific immune function of lymphocytes in the absence of antigen using anti-idiotype reagents. This raises the possibility of the use of such reagents themselves as therapeutic modalities.

Proposed Course of Project: This project is not being pursued at this time due to lack of personnel. If personnel become available the project will be pursued as follows: A) Determine the requirements for triggering antigen-independent responses using monoclonal anti-idiotype including: 1) using the antibody in a form which will produce cross-linking of receptors (Sepharose matrix or covalent cross-linking); 2) providing various forms of T lymphocyte help including helper T lymphocytes which recognize the monoclonal antibody and various lymphokine preparations; and 3) avoiding Fcγ receptor inhibitory effects. B) Determine the mechanisms by which injection of the idiotype in vivo causes expression of the idiotype in an idiotype negative animal.

Publications: None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05062-11 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Application of Rapid Flow Microfluorometry to Cell Biology

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

PI:	J. R. Wunderlich	Senior Investigator	IB, NCI
	S. O. Sharrow	Chemist	IB, NCI
Others:	Members of the Immunology Branch (see text)		IB, NCI

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LAB/BRANCH
Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	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.)*

Using rapid flow microfluorometry (FMF) for analysis and sorting of cells, aspects of the following projects have been supported during the previous year: (1) the expression of IL 2 receptors on normal and malignant cells and on cells from donors treated with IL 2 or undergoing graft-versus-host disease, (2) the characterization of epidermal cells involved with generating immune responses, and (3) analysis of cells transfected with MHC genes. In addition, a user-interactive set of programs has been added to the flow cytometry laboratory to help data processing and presentation.

Project Description

Objectives: The objective of this project is to utilize rapid flow microfluorometry (FMF) to study selected aspects of important problems which would be impossible or extremely difficult to pursue without such technical support.

Methods Employed: Cells have been both analyzed and separated by rapid flow microfluorometry (Fluorescent-Activated Cell Sorter (FACS) and FACS analyzer Becton-Dickinson Electronics Laboratory, Mountain View, CA). These studies involve preliminary treatment of suspensions of viable, dispersed cells with purified, well-characterized fluorescent reagents.

Major Findings: This report summarizes the major thrust of each overall project emphasizing those aspects most heavily supported by the use of rapid flow microfluorometry (FMF).

Bluestone, D. Singer and colleagues. FMF has been utilized to monitor the expression of MHC determinants by transfected cells. Thus, Bluestone et al. have generated hybrid class I MHC molecules by shuffling the genes encoding external domains. By monitoring the level of determinant expression by FMF, they demonstrated that γ-interferon treatment of transfected cells was necessary to boost the expression of hybrid MHC molecules to near-normal levels. Under these conditions their results indicated that MHC recognition by CTL, generated against allodeterminants associated with a mutation in one of the domains, is influenced by interactions between the domains. D. Singer et al. have used FMF to demonstrate that 2 MHC gene constructs, isolated from a miniature swine cosmid library, are expressed as serologically distinguishable cell-surface proteins by transfected mouse L cells.

Shearer, A. Singer and colleagues. This group has utilized FMF to analyze the expression of IL 2 receptors by fresh spleen cells from mice undergoing graft-versus-host (GVH) disease. Loss in the expression of these receptors is apparent as early as one week after induction of the disease and loss is profound by the fourth week. FMF has also been used to analyze the fate of host T cells during GVH. When the disease is induced selectively against class II MHC differences, hosts are still capable of responding to class I alloantigens but not against modified-self antigens--such as TNP-treated syngeneic cells. FMF analysis has demonstrated selective loss of $L3T4^+$ T helper cells in mice with GVH generated by class II differences. Additional studies have demonstrated that Lyt 2^+ T cells can provide adequate help for responses against class I alloantigens.

Waldmann, Nelson, Lotze, Rosenberg and colleagues. These groups have used FMF in conjunction with clinical studies in order to assess the expression of IL 2 receptors on both normal and malignant cells. Members of the Metabolism Branch (NCI) have found a close association between IL 2 receptor expression and infection of human lymphoid cells by adult T-cell lymphotropic virus-I (HTLV-I). Thus, all the populations of leukemic cells from 11 patients with adult T-cell leukemia associated with HTLV-I infection were Tac^+ (expressed IL 2 receptors). In contrast, acute T-cell leukemic populations and lines derived from these cells ($HTLV\text{-}I^-$) did not express IL 2 receptors (Tac^-). Furthermore, nine of 10 populations of Sezary leukemic T-cells, not associated with HTLV, were Tac^-.

FMF has also been used successfully to monitor tumor load in patients with IL 2 receptor positive leukemia cells during therapy with monoclonal anti Tac. FMF has been particularly useful in detecting episodes of early relapse. Members of the Surgery Branch (NCI) previously demonstrated changes in peripheral blood mononuclear cells from cancer patients treated with IL 2. Among the changes, recent FACS analyses have revealed that continuous administration of human recombinant IL 2 caused a 2-12 fold expansion of circulating mononuclear cells, 25-40% of which expressed IL 2 receptors. Further study by 2-color FACS analysis has demonstrated that the Tac^+ cells consist of both T cells and monocytes. Most of the Tac^+ T cells can be accounted for by Leu 3^+ ($OKT4^+$) T cells, which are those commonly associated with "helper" activity. Over 30% of the Leu 3^+ cells bear the Tac antigen, whereas less than 5-10% of the Leu 2^+ ($OKT8^+$) cells (commonly associated with cytotoxic or suppressor cell activities) are Tac^+. Most monocytes which express the M3 antigen are Tac^+.

Berzofsky and colleagues. This group has used FMF to demonstrate that in contrast to general opinion, cells expressing IL 2 receptors can be detected among resting mouse spleen cells. This finding resulted from 2-color FMF, which showed that IL 2 receptors are expressed only by cells among the $L3T4^+$ subpopulation of resting T cells. After nonspecific stimulation in vitro, cells expressing IL 2 receptors occur in both the $L3T4^+$ and Lyt 2^+ subpopulations of T cells. The fraction of resting splenic T cells expressing IL 2 receptors is strain-dependent. The number of IL 2 receptor positive cells is controlled by multiple genes, very likely including at least one gene on chromosome 7.

Katz, A. Singer and colleagues. The expression pattern of cell-surface determinants (phenotype) on murine epidermal cells has been measured by 2-color FMF in order to help assess the state of differentiation and possible function of these cells. The bone marrow derived dendritic cells (Langerhans cells), which are potent antigen presenting cells and accessory cells, express unusually low levels of MHC class I determinents relative to other cells. The expression of class I MHC determinants on Langerhans cells was only 0.09-0.14 as much as on B cells and only 0.016-0.033 as much as on keratinocytes. By contrast, class II MHC expression was 1.4-1.8 times greater than that on B cells. Freshly isolated Thy 1^+ epidermal cells are $L3T4^-$, Lyt 2^- but asialo GM 1^+, Ly 5.1^+--a phenotype consistent with primative T cells. Two cell types which emerge following three weeks of culture with the mitogen, concanavalin A, have phenotypes consistent with natural killer cells (Thy 1^+, asialo GM 1^+, $L3T4^-$) and T-helper cells (Thy-1^+, asialo GM 1^-, $L3T4^+$).

Powell, Sharrow, Stephany, Schultz and colleagues. A major set of data processing programs, APR, has been added to the FACS laboratory. APR involves interactive programs with graphics capabilities which offer easy comprehension, easy use, and wide ranging capabilities. This addition to the FACS lab offers more user-friendly means of data analysis and presentation.

Significance to Biomedical Research and the Program of the Institute: Interaction between IL 2 and its cell surface receptor is essential for normal T cell function. Investigators using FMF technology combined with recently developed monoclonal antibodies against human and murine IL 2 receptors, have identified a variety of changes in IL 2 receptor expression which correlate

with changes in cell function. Thus, peripheral blood cells from patients treated with continuous infusion of IL 2 have increased numbers of IL 2 receptor positive cells and also are unusually responsive to IL 2 in vitro. Genetic regulation of the number of IL 2 receptor positive cells is complex, but FMF analysis of mouse cells from inbred and recombinant inbred strains suggest location of at least one of the genes on mouse chromosome 7. In interpreting the close relationship between HTLV-I infection and IL 2 receptor expression, investigators have noted the possibility that a portion of the HTLV-I genome regulates IL 2 receptor expression and that IL 2 receptor expression on human leukemia T cells may contribute to their uncontrolled growth. In contrast to the potential association of excessive expression of IL 2 receptors in uncontrolled cell growth, loss of IL 2 receptor-positive cells in graft-versus-host (GVH) disease correlates with loss of immune responsiveness. In this regard assessment of IL 2 receptor expression may be of diagnostic value for detecting early signs of immune reactions against the host (e.g., GVH or autoimmunity).

Phenotyping cells by FMF, which detects the level of expression of cell surface differentiation determinants, has been particularly useful in establishing the state of differentiation or maturation of cells found in skin. Both antigen-presenting cells and T cells are common to skin, but their phenotypes suggest a more primitive level of maturation than counterparts in lymphoid organs.

Proposed Course of Project: As in the past, FMF will be used for selected appropriate projects.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05064-10 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic Control of the Immune Response In Vitro

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

PI:	A. Singer	Senior Investigator	IB, NCI
Others:	R. Gress	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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 influence of class I MHC gene products on selection of the L3T4$^+$ T helper cell repertoire was investigated. It was found that the generation of immune responses specific for K^{bm6} class determinants involved L3T4$^+$ T helper cells specific for a composite MHC determinant composed of I-A^b + K^{bm6} determinants. Most interestingly, it was found that the selection of L3T4$^+$ T helper cells specific for IA^b + K^{bm6} composite determinants required intra-thymic expression of I-A^b + K^b composite MHC determinants. In other words the selection of Ia-restricted Th cells specific for mutant class I determinants required expression by intrathymic cellular elements of self-class II and self class I MHC determinants. This is the first example of class I and class II gene complementation involved in the selection of the T cell repertoire.

Project Description

Objectives: The major objective of this project is to examine the specificities involved in the selection of the T helper cell repertoire.

Methods Employed: Purified populations of $L3T4^+$ and $Lyt2^+$ T cells were isolated by negative selection and stimulated in vitro with mb6 stimulator cells and assayed for the generation of anti-K^{bm6} CTL. $Lyt2^+$ T cells were always from normal B6 mice whereas $L3T4^+$ T cells were obtained from normal, chimeric, and thymus-engrafter experimental mice.

Major Findings: The generation of $L3T4^+$ Th cells functional in anti-bm6 responses requires intra-thymic expression of both IA^b and K^b determinants.

Significance to Biomedical Research and the Program of the Institute: Understanding the basis of T repertoire selection will make it ultimately possible to manipulate the specificities of T helper cells.

Proposed Course of the Project: Our immediate goals are to map within the K^b molecule the exact site required for selection of K^{bm6}-specific $L3T4^+$ T helper cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05067-11 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Mechanisms of In Vitro Cellular Immune Responses

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

PI:	S. Shaw	Senior Investigator	IB, NCI
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	M. Sanders	Medical Staff Fellow	IB, NCI
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	R. Gress	Senior Investigator	IB, NCI
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Timothy A. Springer, Ph.D., Department of Membrane Immunochemistry, Dana Farber Cancer Institute, Boston, MA 02115

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have explored CTL-target interactions using well characterized cloned human CTL. Conjugate formation between these CTL and many antigen-negative targets is almost as efficient as with specific target cells, but does not lead to target cell lysis. These findings suggest that, on specific target cells, adhesion by antigen-independent pathways may occur concurrently with or precede antigen recognition. Our MAB inhibition studies using individual MAB and mixes: 1) demonstrate that CD18, CD2, and LFA-3 are involved in antigen-independent conjugate formation; and 2) suggest that the CD2 and LFA-3 molecules are involved in one pathway and the CD18 molecule is involved in a distinct pathway. Confirmation of the existence of distinct pathways was provided by findings that CD18-dependent adhesion requires cations and is temperature-sensitive while CD2- and LFA-3-dependent adhesion does not require cations and is temperature-insensitive. Together with previous data, these studies strongly suggest that CD2 on the effector may interact with LFA-3 as its ligand on targets. Purification of the LFA-3 protein is in progress. Cloning of the LFA-3 gene is being attempted by "shotgun" transfection and by oligonucleotide probing of a cDNA.

Project Description

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of cell surface structures in T cell recognition, and the mechanisms by which these structures control immune responses. Although the hypotheses generated are based on insight from many areas of expertise and model systems with a variety of species, the experimental work is focused on human studies. Because of the ethical and logistical considerations which limit in vivo studies in humans, it has been (and is) crucial to develop good in vitro models of human immune responses. The model system used most extensively in recent studies has been interaction between HLA-DPw2 specific cytotoxic T cell clones and their potential targets. Over the tenure of this project emphasis has shifted from the role of HLA genes in regulating immune responses towards the role of other cell surface structures which are critical to T cell recognition--particularly those which are involved in establishing and maintaining close contact between T cells and potential target/stimulator cells.

Methods Employed: Human peripheral blood mononuclear cells are obtained from donors, purified by density separation, and cryopreserved. The HLA antigens of such cells are characterized by serotyping (at the NIH clinical center or via contract NO1-CB33935). Allogeneic in vitro mixed lymphocyte cultures are established between carefully selected combinations of donors, and weak responses are often amplified by in vitro restimulation. For higher resolution analysis of the specificity and function of individual T cells, single cell cloning has been performed by limiting dilution and subsequent expansion in the presence of specific antigen and IL2. Primed cells are frozen in large batches and thawed as necessary to provide standard reagents. Proliferation of such cells in response to stimulator cells is measured by ^{3}H-thymidine incorporation. Cytotoxic activity is analyzed by short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines. Analysis of cell surface phenotype has been accomplished using a variety of monoclonal antibodies and binding analyzed by flow cytometry. Conjugate formation between CTL and "targets" is measured by a two color fluorescence technique which exploits flow microfluorometry to objectively enumerate conjugates; the combinations of dyes used in this assay has been modified in our laboratory to avoid surface alteration of cells which could perturb the physiologic interactions being observed. "Shotgun" transfection of human genomic DNA into murine cells is accomplished by routine purification of DNA from lymphoblastoid cell lines, and cotransfection with tk into a highly transfectable L cell subline. Cells transfected with the gene of interest have been identified by indirect rosetting or by cell sorting on a flow microfluorometer. Isolation of cell surface molecules has been achieved by physical separation of plasma membranes, followed by detergent solubilization of those membranes and immunoaffinity purification on appropriate columns of immobilized monoclonal antibodies.

Major Findings: We have explored CTL-target interactions using well characterized cloned human CTL and a wide variety of target cells. Last year's annual report described our optimization of the assay system for conjugates and findings on strength of conjugates, kinetics of formation, and molecules involved in conjugate formation (CD18, CD2, LFA-3). Of particular interest has

been the finding that conjugate formation between these CTL and many antigen-negative targets is almost as efficient as with specific target cells, but does not lead to target cell lysis. Such antigen-independent conjugate formation could be either an in vitro artifact, or an important biologic process involved in T cell recognition. Several lines of argument have convinced us that such conjugate formation is not artifact, but is rather a central process in T cell recognition. 1) Antigen-independent conjugate formation has been seen, in varying degrees, in a variety of assay systems in which it has been looked for (in our lab and previously in other labs). Our studies have demonstrated that antigen-independent conjugates are formed in the absence of added lectin, that they are formed even when using T cells that have only been in culture for 7 days (rather than long term cultured T cell clones) and that they can be formed with a variety of target cell types. 2) The three cell surface molecules which are critical to antigen-independent conjugates are essential to cell-mediated cytotoxicity and to conjugate formation with antigen-positive target cells. 3) Theoretical considerations predict that antigen-independent conjugate formation is essential to achieving sensitivity of T cell recognition. In light of these three kinds of evidence for the relevance of antigen-independent conjugate formation, we have placed a major emphasis on understanding this phenomenon.

The combination of two lines of evidence has demonstrated the existence of two pathways of antigen-independent conjugate formation. 1) When mixes of monoclonal antibodies (αCD18, αCD2, αLFA-3) were assayed for inhibitory capacity, additive (or even synergistic) effects were observed with a combination of αCD18 plus either αCD2 or αLFA-3. However, the mix of αCD2 and αLFA-3 was no more inhibitory than αLFA-3 alone. These results are consistent with the hypothesis that the CD2 and LFA-3 molecules are involved in one binding pathway while the CD18 molecule is involved in a distinct pathway. If CD2 and LFA-3 participate in the same pathway, then antibodies against both would not have additive effects at optimal concentrations because either alone would block the postulated CD2/LFA-3 pathway. In contrast, αCD2 or αLFA-3 will have enhanced effectiveness when mixed with αCD18, because each MAB in the mix is inhibiting a different pathway. 2) Using monoclonal antibodies to selectively block one pathway, it was possible to analyze requirements for each of the pathways individually. CD18-mediated conjugate formation (which remains after αCD2 or αLFA-3 inhibition) is temperature-sensitive and depends on a cation which is bound by EDTA but not EGTA--most likely magnesium. CD2- and LFA-3-dependent conjugate formation (which remains after αCD18 inhibition) is temperature-insensitive and cation-independent.

These studies which indicate that CD2 on the effector and LFA-3 on the target participate in the same pathway of conjugate formation strongly suggest that CD2 on the effector may interact with LFA-3 as its ligand on targets. Consequently, we have begun to vigorously pursue other studies of this little known molecule LFA-3. Efforts are in progress to clone the LFA-3 gene; two different approaches are being tried in parallel. The first approach is by "shotgun" transfection of genomic human DNA into murine L cells and selecting for the rare L cell which has integrated and expressed the LFA-3 gene (modelled after successful pioneering studies by Herzenberg's lab and Ruddle's lab). Despite success in achieving high frequency of transfectants, no LFA-3 transfectants

were selected by an immunorosetting assay, and efforts at selection by sequential fluorescent activated cell sorting are in progress. The alternate method for gene cloning is by protein isolation, limited amino acid sequence determination, and screening cDNA libraries with oligonucleotide probes. Efforts at protein isolating are in progress. We believe that this will be facilitated by our discovery that the LFA-3 molecule is expressed on human erythrocytes (see below), which are readily available as a starting material for purification.

We have also determined that LFA-3 is expressed on human erythrocytes (but αLFA-3 does not cross-react on sheep erythrocytes). This observation prompted studies of monoclonal antibody inhibition of human T cell rosetting with human and sheep erythrocyte. Those studies indicate that αCD2 inhibits rosetting with both kinds of erythrocytes, while αLFA-3 inhibits only human erythrocyte rosettes. These findings strongly support the hypothesis that LFA-3 is a ligand for CD2.

To study the role of CD18 on the target cell side of CML, we have investigated CML and conjugate formation between a cytotoxic T cell clone and EBV transformed lymphoblastoid cell lines (LCL) derived from seven patients with heritable CD18 deficiency (which express from 5% to less that 0.2% of the normal amount of cell surface CD18). Studies of three DPw2-positive CD18 deficient LCL demonstrate that they do not differ from other DPw2-positive LCL with respect to: 1) susceptibility to lysis by this DPw2-specific CTL; 2) ability to serve as cold target inhibitors of this cell mediated lysis; 3) susceptibility of lysis to blocking by monoclonal antibodies against CD18, LFA-2 (CD2), LFA-3, T3 (CD3), T4 (CD4), and DP; 4) ability to form conjugates with the CTL. Furthermore, studies of one DPw2-negative CD18 deficient LCL indicate that, like other LCL, it forms antigen-nonspecific conjugates with this LTL clone. These experiments are consistent with the lack of a significant functional role of CD18 on the target cell side in conjugate formation with and lysis by a cytolytic T lymphocyte clone from a normal donor.

Significant to Biomedical Research and the Program of the Institute: The problems addressed in this project are central to the understanding of how the immune system surveys the body to detect foreign antigen and to eliminate cells which express those foreign antigens. Cell-cell adhesion is essential for many immunologic functions including interaction of cytotoxic T lymphocytes (CTL) with their targets. Our studies begin to define important features of the cell biology of this interaction and to characterize the molecular pathways involved. Defects in these pathways have important clinical consequences--as illustrated by the clinical syndrome due to CD18 deficiency. We believe that other alterations in these pathways may predispose to clinical syndromes which remain to be defined. Furthermore, pharmacologic manipulation of these pathways may be a therapeutic benefit to patients with abnormal immune status.

Our studies prompt the important hypothesis that LFA-3 is the cell surface ligand for CD2. This previously known molecule CD2 (also known as T11, LFA-2 and the sheep rosette receptor) is being studied in many laboratories as a critical regulator of immune function. This raises the possibility that LFA-3, structural homologs, or peptide fragments of it, could be used as a potent

pharmacologic agent: 1) in enhancing the natural immune response of patients with decreased responses; or 2) in reducing the immune responses of patients with overactive immune responses.

Proposed Course of Project: Efforts will continue to define the cell biology of antigen-independent conjugate formation. A variety of important parameters remain to be examined. For example: What cell surface and intracellular molecules are recruited into the zone of interaction between effector cells and target cells? What pharmacologic agents interfere with each pathway of adhesion? and how does that help us understand the molecular interactions? What regulates the termination of the effector-target interaction, particularly for antigen-independent conjugates?

We will continue to pursue the cloning of the LFA-3 gene to understand its molecular structure. Isolation of the LFA-3 protein will be necessary for one of the approaches to cloning and will make possible molecular studies of the binding interactions which occur.

Publications:

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Hoffman, R. W., Quinones, R. R., and Shaw, S. 1985. Anti-T3 antibody both activates and inhibits the cytotoxic activity of human T cell clones. Behring Inst. Mitt. 77:30-38.

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Perez, P., Hoffman, R. W., Shaw, S., Bluestone, J. A., and Segal, D. M. 1985. Specific targeting of CTL by anti-T3 linked to anti-target cell antibody. Nature 316:354-356.

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Shaw, S. 1985. Book review. Methods in enzymology. Volume 108. Immunochemical Techniques Part G. Anal. Biochem. 147:265-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05069-10 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Expression of Ia Antigens on Functional Cell Subpopulations

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

PI:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
Others:	D. H. Sachs	Chief, Transp. Biol. Sec.	IB, NCI
	D. H. Lynch	Investigator	IB, NCI
	A. Finnegan	Senior Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

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Marseille, France

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

The responses of both cloned and heterogeneous T cell populations to mitogens and specific antigenic stimuli were found to require the participation of accessory or antigen presenting cells which express Ia (I region associated) determinants. A series of T cell clones which recognized the same antigenic molecule in association with a given I-E molecule were studied for their susceptibility to inhibition by a panel of 15 different anti I-E monoclonal antibodies specific for determinants on the same I-E molecule. The results demonstrated that different T cell clones appear to recognize antigen in association with distinct determinants or conformations on the same I-E molecule. The responses of T cell clones to the mitogen Mycoplasma arthritidis supernatant (MAS) were analyzed. It was determined that approximately 15% of the clones tested responded to MAS when presented in the context of I-E bearing antigen presenting cells, and that this reactivity was independent of the primary specificity or MHC restriciton of those clones to conventional antigens. The ability of different Ia expressing populations to present to cloned T cells was evaluated. All of the clones tested were able to respond to adherent cell-containing irradiated stimulating cells or to mitomycin treated purified resting B cell populations. In contrast, only a subpopulation of cloned T cells was responsive to LPS activated B cell blasts, in spite of the enhanced Ia expression by these B cell blasts. These findings indicate that the signals required for T cell activation may vary among T cell clones, and that distinct Ia bearing antigen presenting cells may be competent for presentation to different T cells. Additional studies employing cloned Ia^+ B cell lymphomas and hybridomas also indicate a heterogeneity among T cells in their responsiveness to these populations.

Project Description

Objectives: Ia antigens are serologically demonstratable cell surface determinants which appear to play important roles in cell-cell interactions. The objective of this study is to investigate the role these Ia determinants play in cellular interactions involved in T cell-mediated or T cell-dependent responses. The T cell proliferative response to mitogens and to soluble antigens, and T cell-dependent in vitro antibody responses are being studied. Studies employing monoclonal antibodies and cloned T cell populations have been directed at defining the functional importance of different determinants on the same Ia molecules.

Methods Employed: Cloned T cell populations have been generated by in vivo priming with soluble antigens, in vitro re-stimulation, and limiting dilution. These clones include I-A or I-E restricted T cells specific for antigens including KLH, GLPhe, and GLleu. Monoclonal anti-Ia antibodies have been employed including a panel of 15 anti-I-E^k antibodies specific for different domains on the I-E^k molecule as defined by competitive binding. Antigen-presenting cells have included both heterogeneous spleen cell populations and cloned B cell lines.

Major Findings: Cloned T cells specific for the antigens GLPhe, GLleu, KLH, and cytochrome C were generated and were shown to be both antigen-specific and under the control of complementing immune response genes in the I-A and I-E subregions. It was shown that individual clones had different patterns of susceptibility to inhibition by a panel of anti-I-E antibodies, suggesting that different T cell clones recognize antigen in association with different epitopes on the same Ia molecule. The supernatant of Mycoplasma arthritidis has previously been shown to be mitogenic for T cell populations. A panel of cloned T cells of varying MHC restriction and antigen specificity was employed to study reactivity to MAS. When T cell depleted populations of antigen presenting cells were employed, it was found that approximately 15% of T cell clones were reactive to MAS and were reactive only when I-E expressing antigen presenting populations were employed. The functional role of I-E products in these responses was confirmed by the inhibition of cloned T cell response by anti I-E monoclonal antibodies. Responsiveness to MAS + I-E product was independent of the primary antigen specificity or MHC restriction specificity of the responding cloned T cells.

In order to determine the requirements for T cell activation by Ia^+ antigen presenting cells, different Ia^+ populations were compared for their ability to trigger proliferative responses in cloned T cells. All of the T cell populations tested were Ia restricted in their responses, and all T cells responded to irradiated adherent cell-containing stimulating populations or to mitomycin C treated purified resting B cell populations. In contrast, some but not other cloned T cells were responsive to LPS activated B cell blasts of appropriate Ia phenotype. In addition, the use of monoclonal B cell lymphoma and hybridoma populations revealed a heterogeneity among T cells, with some but not other clones responsive to these B cell populations. These findings indicate that there is a heterogeneity in T cell activation requirements, such that some but not other Ia expressing populations are competent to activate a given cloned T cell.

The role of antigen processing in presentation to T cells was studied through the use of chloroquine treatment of antigen presenting populations. This treatment, which serves to inhibit lysosomal processing, interfered with the ability of presenting populations to process and/or present soluble antigens to cloned T cells. In contrast, the responses of the same or different cloned T cells to self (autoreactive) or allogeneic (alloreactive) Ia determinants was was not influenced by treatment with low concentrations of chloroquine. These findings indicate that the in vitro antigen processing requirements for foreign soluble antigens are distinct from the requirements for presentation of intrinsic cell surface Ia determinants.

Significance to Biomedical Research and the Program of the Institute: I region gene products play a significant role in regulation of immune responses. A basic understanding of the expression, and perhaps the functional role of one class of I region gene products should provide insight into the mechanisms of antigen presentation and T cell recognition in immune responses. The findings to date suggest that discrete sites on Ia molecules are differentially important in antigen recognition by different T cell clones. This may reflect a heterogeneity in the sites on Ia molecules available for interacting with specific antigens and/or a heterogeneity in the Ia sites which can be recognized by T cells. The finding that individual T cell clones differ in their responsiveness to Ia^+ B cell populations has substantial significance in understanding the interaction between T cells and B cells. The possibility that, for example, certain T helper populations are capable of interacting with resting, but not activated, B cells would have important implications for the understanding of T cell regulation in B cell responses.

Proposed Course of Project: Cloned lines of antigen-presenting cells have been generated which express I-A and I-E products of several different haplotypes. These lines and variants derived from them will be utilized to further compare the activation and recognition requirements of different cloned T cell populations.

Publications:

Lynch, D. H., Cole, B. C., Bluestone, J. A., and Hodes, R. J.: 1986. T cell responsiveness to Mycoplasma arthritidis supernatant (MAS) is clonally expressed. Eur. J. Immunol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05086-08 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Immune Response Gene Regulation of the Immune Response In Vitro

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

PI:	R. J. Hodes	Chief, Immunotherapy Section	IB, NCI
Others:	D. H. Sachs	Chief, Transp. Biol. Sec.	IB, NCI
	A. Finnegan	Investigator	IB, NCI

COOPERATING UNITS *(if any)*

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Immunology Branch

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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.)*

The function of accessory cells in primary and secondary in vitro antibody responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L is under the control of immune response (Ir) genes which map to I-A. The expression of Ir gene function by B cells is related to the B cell activation pathway; Ir gene function is expressed by B cells activated under conditions involving MHC-restricted T-B interaction. In vitro augmented primary and secondary responses to TNP-nuclease (TNP-NASE) have also been established and documented to be under the control of H-2 linked Ir gene(s) mapping to the I-B subregion. For these responses, accessory cell function was shown to be under Ir gene control. Recent data employing monoclonal anti-Ia reagents have suggested that genes in the I-A subregion may also be involved in regulating responses to TNP-NASE.

In order to further analyze the genetic regulation of T cell responses to NASE, a series of cloned lines were generated in BALB/c ($H\text{-}2^d$) as well as ($H\text{-}2^b$ x $H\text{-}2^a$)F_1 T cells. Individual clones were restricted to recognizing NASE in the context of either $A_\alpha A_\beta$ or $E_\alpha E_\beta$ 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.

Project Description

Objectives: The major objective of this project is to investigate the mechanism of genetic regulation of antibody responses. Initial studies identified the cellular level of Ir gene expression for the in vitro responses to TNP-(T,G)-A--L, TNP-(H,G)-A--L and TNP-NASE. Subsequent studies assessed the Ir gene regulation of activation of defined B cell subpopulations. Current studies are employing cloned populations of antigen specific T cells and synthetic antigenic peptides to analyze the structural features which determine antigenicity as well as to analyze the mechanisms underlying selective association of a given antigenic determinant with a specific Ia restricting element.

Methods Employed: The methods employed have been described in detail. See project No. Z01 CB 05064-10 I.

Major Findings: The in vitro primary and secondary antibody responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L are under strict H-2 linked gene control by genes mapping to the I-A subregion. When B cells are activated by a pathway requiring H-2 restricted T-B interaction, B cell function is found to be under Ir gene control. In contrast, in responses requiring no restricted T-B interaction, B cell function was not under Ir gene control.

In vitro TNP-specific responses to TNP-conjugated Staphylococcal nuclease (TNP-NASE) were generated by spleen cells from NASE-primed mice. These responses were T-cell and accessory cell-dependent, and under H-2-linked Ir gene control, with strains of the $H\text{-}2^a$ haplotype being responders and $H\text{-}2^b$ strains nonresponders. Ir gene control mapped to I-B and was not explained by complementing genes in I-A and I-E/C. Cell fractionation experiments have shown that accessory cell function is under Ir gene control for the response to TNP-NASE.

Monoclonal T_H cell populations specific for NASE have been generated in BALB/c ($H\text{-}2^d$) and (B10xB10.A)F_1 ($H\text{-}2^b \times H\text{-}2^a$) genotypes. Of the BALB/c clones tested, individual clones show MHC restriction for either I-A or I-E products. 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.

Significance to Biomedical Research and the Program of the Institute: Genetic control of immune responses has been demonstrated in widely studied systems, including those responses to biologically "natural" antigens including allergens, viral determinants, and tumor antigens. In order to understand the mechanism of differential reactivity and susceptibility to these natural stimuli, the mechanism of Ir gene regulation of responses to defined stimuli may provide informative insights.

Proposed Course of Project: Cloned T cell populations 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.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05088-08 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

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	IB, NCI
Others:	M. Moser	Fogarty Fellow	IB, NCI
	I. Iwasaki	Fogarty Fellow	IB, NCI
	F. Hakim	Guest Worker	IB, NCI
	C. S. Via	Medical Staff Fellow	IB, NCI
	M. Fukuzawa	Fogarty Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	4.0	1.5

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

B

SUMMARY OF WORK *(Usa 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.

GVHID is accompanied by loss of ability to produce IL 2 and in loss of expression of IL 2 receptors. This IL 2 loss was observed in both class I and II and only to a limited extent in class II only GVHID. Recovery of immune function from GVHID was preceeded by recovery of IL 2 receptor expression and IL 2 production.

GVHID was used to abrogate natural resistance to bone marrow grafts in F_1 hybrid mice, and recognition of host only class II antigens is required to abrogate natural resistance.

GVH can also induce immune deficiency in more primitative elements of the lympho-hematopoietic system, including thymus and bone marrow functions.

Project Description

Objectives: The purpose of this project is to investigate the phenomenon of immunosuppression induced by or associated with a graft vs. host reaction, the immunogenetics associated with the resistance in some strain combinations of the graft vs. host associated suppression, and to attempt to establish whether this immunosuppression is correlated with autoimmune states and/or the development of immune deficiency states following a graft vs. host reactions.

Methods Employed: F_1 hybrid mice of various strains were injected intravenously with from 1 to $40x10^6$ F_1, parental, or allogeneic spleen cells. Inbred mouse strains were also injected with allogeneic cells. At various times after injection, the spleens of the injected F_1 mice were sensitized in vitro against: (a) parental or F_1 syngeneic cells modified with TNBS; or (b) allogeneic spleen cells. The effector cell actively generated 5 days later was tested on the appropriate ^{51}Cr-labelled target cells. F_1 mice were immunized to H-2 alloantigens or H-1 antigens prior to or at the same time that the F_1 mice were inoculated with parental cells. Chimera GVH mice were prepared by injecting F_1 mice with parental spleen cells, followed by irradiation and reconstitution with F_1 bone marrow cells. IL 2 production was measured by culturing spleen cells with ConA, harvesting the supernatants, and assaying the IL 2 activity on the HT2 cell line. IL 2 receptors were investigated using FMF by culturing spleen cells with the anti-IL 2 receptor monoclonal antibodies 7D4 and 3C7. The test for thymic function in GVH mice involved whole-body lethal γ-irradiation (with or without parental thymic transplantation) of the GVH mice followed by bone marrow reconstitution and the in vitro testing of recipients' splenic T cell immune function. Stem cell function was tested by inoculation of T cell depleted bone marrow from GVH mice into lethally irradiated recipients, followed by testing of the repopulating recipients, spleens for donor cell proliferation five days later or of testing the recipients' spleens eight-to-twelve weeks later for T cell immune function.

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 $L3T4^+$ T helper cell function and of $L3T4^+$ 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 $L3T4^+$ and $Lyt2^+$ T helper cell function. The induction of class II only GVHID required only $L3T4^+$ parental cells, whereas induction of class I + II GVHID required $L3T4^+$ and $Lyt2^+$ cells.

Lymphocytes from mice that are immunosuppressed by GVHID produce reduced amounts of IL 2 and lose their IL 2 receptors. IL 2 receptor expression and IL 2 production began to reappear 8 weeks after GVHID induction and recovery of immune funciton began to reappear at 12 weeks. Active suppression of IL 2 reception was demonstrated by mixing spleen cells from normal and GVH mice.

The recovery of IL 2 receptors and immune function can be attributed to donor T cell repopulation of the GVH host mice.

Protection against GVH-suppression could also be induced by immunizing the F_1 mice to H-2 alloantigens prior to induction of suppression. This protection 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 were lost whereas MHC allogeneic responses remained intact. Furthermore, the developmental stages of AIDS and class II GVHID result in a loss of $CD4^+$ and $L3T4^+$ 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.

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.

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 H-2 self-restricted 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.

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.

Significance to Biomedical Research and the Program of the Institute: The graft vs. host (GVH) reaction and possibly Hh-type reactions are important complicating factors which affect the success of hemopoietic transplantation. Since several recent cases of human marrow transplantation involving treatment of marrow with anti T cell reagents have resulted in failure to engraft, it may be that a chronic GVH is necessary to promote engraftment. Our GVH abrogation of natural resistance to marrow grafts may be relevant for this.

Furthermore, persisting GVH reactions may be associated with autoimmune disease and the development of tumors. The observations: (a) that GVH reactions can be elicited with low numbers of lymphocytes in immunocompetent adult mice (previous reports have been limited to the demonstration of GVH in neonates or immunosuppressed animals); (b) that these GVH reactions lead to severely impaired T-cells immune functions; and (c) that such GVH reactions can be overcome by host resistance mechanisms are potentially of fundamental relevance in: (1) understanding the possible complications resulting from hemopoietic grafting; (2) investigating the significance of a GVH-induced suppressed immune system in the development of autoimmune and neoplastic disease; and (3) understanding natural resistance systems as they may be relevant in surveillance against disease and neoplasms. It is possible that our murine model has genetic and mechanistic relevance for SCID. It is also possible that GVH-associated immunosuppression may be a good mechanistic model for the development of AIDS. Models of this type are consistent with the Program of the Institute.

Proposed Course of Project: We shall continue to investigate many aspects of the phenomenon including: (a) the genetics of the F_1 and parental cells involved; (b) the determinants recognized on the F_1 cells; (c) the mechanistic aspects of both the GVH and the suspected Hh component involved; (d) other immune functions which may be impaired including antibody production, delayed hypersensitivity, skin graft rejection, T-cell proliferative responses, and natural killer cell activity; (e) the long-term effects of the GVH including survival and the development of autoimmune disease and tumors; (f) which combinations of allogeneic cells and hosts can lead to GVH-associated immunosuppression; (g) analysis of different populations of cells involved in the induction of and protection by antibody against GVH-associated immunosuppression; (h) whether GVH-associated suppression is a useful model for AIDS; (i) analysis at the molecular level of the failure of T cells from GVH mice to express IL 2 receptors; (j) analyses of the thymic and stem cell defects of GVHID mice.

Publications:

Shearer, G. M., Moser, M., Iwasaki, T.: 1985. Graft-versus-host-induced T cell immunodeficiency in mice. Immunol. Rev. 88:135-151.

Lang, P., Dardenue, M., Savino, W., Moritz, S., and Shearer, G.: 1986. Cytotoxic T cell response and thymic hormonal dysfunction in graft-vs-host mice. J. Immunol. 136:1999-2004.

Shearer, G. M., and Moser, M.: 1986. AIDS as a consequence of Ia antigen recognition: a closer look. Immunol. Today 7:34-36.

Joseph, L. J., Iwasaki, T., Malek, T., and Shearer, G. M.: 1985. Interleukin 2 dysfunction in mice undergoing a graft-versus-host reaction. J. Immunol. 135:1846-1850.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05099-06 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Synergistic Effects of Murine Cytomegalovirus and Graft-versus-host Reaction

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

PI:	G. M. Shearer	Senior Investigator	IB, NCI
Others:	J. Titus	Chemist	IB, NCI
	C. Via	Medical Staff Fellow	IB, NCI

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J. D. Shanley, V.A. Hospital, Newington, CT

LAB/BRANCH

Immunology Branch

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Mice injected with sublethal doses murine cytomegalovirus (MCMV) exhibit rapid and dramatic changes in their ability to generate in vitro cytotoxic T lymphocyte responses to hapten-self and to alloantigens, and to produce IL 2 and express IL 2 receptors. Within three days after intraperitoneal injection of MCMV, the CTL responses to hapten-self and alloantigens are abrogated or severely reduced. This is followed by rapid recovery to a normal level of CTL potential. Injection of F_1 hybrid mice with either MCMV or parental spleen cells (graft-versus-host reaction - GVHR) resulted in rapid and severe immunosuppression. Inoculation of either the virus or parental cells were selected so that they would be below the threshold for severe immunosuppression. However, when these two inocula were combined, severe immunosuppression was observed. Furthermore, injection of F_1 mice with parental lymphocytes that recognize only class I MHC determinants does not result in immune suppression. However, a combination of class I recognition and MCMV infection results in profound immune suppression. Infection of host mice with MCMV prior to induction of GVH resulted in augmented immune suppression, whereas infection of donor mice with MCMV before induction of GVH resulted in reduced immune suppression. 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.

Project Description

Objectives: The purpose of this project is to investigate the immunological and genetic effects of MCMV infection and of MCMV infection plus a GVHR and for HVGR on (a) acquired T cell immunity to hapten-self antigens and alloantigens; and (b) on natural resistance to MCMV infection and the GVHR. Since certain mouse strains are relatively resistant to MCMV and to parental T cell-induced GVHR, it will also be the purpose of this study to investigate the role of H-2-linked and non-H-2-linked genetic effects of resistance and susceptibility to MCMV, to GVHR and to a combination of MCMV and allogeneic cells.

Methods Employed: Sublethal doses of MCMV (prepared from salivary glands of infected mice) were injected intraperitoneally into various inbred and F_1 hybrid mouse strains. Also F_1 mice were injected intraveneously with known concentrations of parental spleen cells, and F_1 mice were also injected with MCMV plus parental cells. The T cell immune potentials of injected and control mice were tested by in vitro sensitization to hapten-self and allo-antigens, and the CTL activity was determined 5 days later using the ^{51}Cr-release assay. Changes in IL 2 receptor expression was determined by Flow Microfluorometry, using the 7D4 murine anti-IL 2 receptor monoclonal reagent. IL 2 production was measured on the HT2 cell line.

Major Findings: The injection of sublethal doses of MCMV resulted in rapid suppression of CTL potential to both hapten-self and allogeneic antigens (within 3 days). This was followed by recovery (by around 7 days), and augmented CTL activity as detected by the hapten-self and not by the allogeneic CTL systems (days 9-13). The injection of F_1 mice with doses of MCMV plus parental spleen cells each of which alone did not drastically reduce CTL potential, resulted in synergistic effect which abrogated CTL potential. This synergistic effect of GVHR and MCMV was most dramatically demonstrated using parent into F_1 in which only class I MHC antigens are recognized. Under such conditions GVH-associated immune suppression is never observed. However, when administered in conjunction with MCMV, severe suppression was observed.

The MCMV-GVHR synergy model was further analyzed by infecting GVH donor or host with MCMV prior to induction of GVHR. The results indicated that immune suppression: a) was more severe when host was infected prior to GVH-induction; and b) was less severe when donor was infected prior to GVH-induction.

Infection with MCMV results in depressed IL 2 production and in some loss of IL 2 receptors. The synergistic effect of sub-threshold doses of MCMV and GVHR were clearly demonstrated by drastic reduction in IL 2 production and IL 2 receptor expression.

The simultaneous inoculation of MCMV and parental T cells also results in interstitial pneumonitis, which does not occur when either insult is given alone. Bronchoalveolar lavage of the MCMV-GVH indicated that the lungs of the mice contained relatively large numbers of donor-derived T lymphocytes. In contrast, donor-derived T cells were not found in the lungs of GVH mice that had been infected with MCMV.

Significance to Biomedical Research and the Program of the Institute: Cytomegalovirus infection is one of the major problems currently facing human bone marrow transplantation, and may become critical in patients undergoing a chronic GVHR. An understanding of the genetic and mechanistic paramaters involved in resistance and susceptibility to CMV in the murine model, the immunosuppression associated with CMV infection, and the possible synergistic effects of CMV infection and chronic GVH should be valuable for both basic and clinical purposes.

Proposed Course of Project: We plan to attempt to introduce immune suppression in allogeneic combinations of mice that differ at minor H loci only using the combinaiton of MCMV infeciton and minor H locus recognition. We also plan to use a marrow transplant model in which mice will be lethally irradiated and grafted with allogeneic marrow (major or minor H locus differences), with and without MCMV infeciton. We shall monitor immune function in the chimeras as well as look for signs of GVH disease. We plan to investigate further the interstitial pneumonitis model.

Publications:

Grundy, J. E., Shanley J. D., and Shearer, G. M.: 1985. Augmentation of graft-versus-host reaction by cytomegalovirus infection resulting in interstitial pneumonitis. Transplantation 39:548-553.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05100-06 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Role of HLA Genes in Human Disease

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

PI: S. Shaw Senior Investigator IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	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.)*

None performed.

Project Description

Major Findings: None in this period.

Proposed Course of Project: To be reactivated when critical insights are achieved which allow our laboratory to make important contributions in this area.

Publications:

Hoffman, R. W., S. Shaw, L. C. Francis, M. G. larson, R. A. Petersen, L. T. Chylack, and D. N. Glass. 1985. HLA-DP (SB) antigens in pauciarticular juvenile rheumatoid arthritis with iridocyclitis. J. Rheumatol. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05101-06 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Definition of Human Histocompatibility Antigens

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

PI:	S. Shaw	Senior Investigator	IB, NCI
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	E. Gugel	Biological Lab Tech	IB, NCI
	K. Kelly	Investigator	IB, NCI
	E. Long	Investigator	NIAID
	R. Sekaly	Investigator	NIAID

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1.5	0.5	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Using T cell recognition (primarily cell-mediated lysis) we have continued to probe the complexities of HLA alloantigens. The model system in which recognition is being analyzed is analysis of the specificity of a panel of 10 previously derived cytotoxic T lymphocyte (CTL) clones. The present studies clearly indicate that the way T cells "see" DP is rather more complex than one might have predicted. Studies of these CTL clones on previously derived HLA-mutant cell lines indicate heterogeneity in specificity among the CTL clones; specifically some clones distinguish between $DPw2^{+}$ DR^{-} LCL targets which have subsequently been shown to differ in expression of DRα mRNA. These data suggest that DRα contributes to recognition by some "DPw2-specific" CTL clones, perhaps because they recognize: a) DRα in a DPw2-restricted fashion, or b) a heterodimer formed by DRα and DPw2β. Additional HLA-mutant LCL have been generated using the novel approach of using CTL themselves as the agent used for negative selection. More than 25 mutants have been successfully isolated. Molecular analysis of these mutants is in progress and suggests the important finding that the limited polymorphism of the DPα chain (between DWw2 and DPw4) is nevertheless critical for T cell recognition.

Project Description

Objectives: Genes of the HLA region are important in tissue transplantation, immune regulation and regulation of susceptibility to a variety of diseases. Consequently, it is crucial to define the gene products of the HLA region. In each of the foregoing areas of importance of HLA, T cell recognition plays a central role. Therefore, we believe that T cells are the most sensitive probe for defining functionally important details of the HLA region. Therefore, we are studying T cell recognition of HLA antigens both to define additional genes (or epitopes) recognized by T cells and to understand the details of that recognition. Ramifications of such observations will be pursued both in the directions of their role in human disease (AR05100) or for their importance in understanding basic mechanisms of immune recognition (AR05067).

Methods Employed: Human peripheral blood mononuclear cells are obtained from donors, purified by density separation, and cryopreserved. The HLA antigens of such cells are characterized by serotyping (at the NIH clinical center or vial contract NO1-CB33935). Allogeneic in vitro mixed lymphocyte cultures are established between carefully selected combinations of donors, and weak responses are often amplified by in vitro restimulation. For higher resolution analysis of the specificity and function of individual T cells, single cell cloning has been performed by limiting dilution and subsequent expansion in the presence of specific antigen and IL2. Primed cells are frozen in large batches and thawed as necessary to provide standard reagents. Proliferation of such cells in response to stimulator cells is measured by ^{3}H-thymidine incorporation. Cytotoxic activity is analyzed by short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines. Analysis of cell surface phenotype has been accomplished using a variety of monoclonal antibodies and binding analyzed by flow cytometry. Mutant cell lines have been generated by mutagenesis with gamma-irradiation or chemical mutagens, and the survivors selected for resistance to lysis by cytotoxic T cells. Molecular genetic analysis of induced mutations has been performed by us with an RNAase protection assay to detect very limited mutational changes and by our collaborators by conventional Northern blot analysis. Transfection of cell lines with isolated genes has been performed by our collaborators.

Major Findings: Three lines of investigation have been pursued, all of which relate to somatic cell genetic approaches to refine our understanding of T cell recognition of DP antigens. First, we have continued to study T cell recognition of HLA-mutant LCL previously derived by our collaborator Dr. R. DeMars. In order to understand details of T cell recognition of the HLA-DPw2 molecule, eight allospecific cytotoxic T cell (CTL) clones derived from DPw2-specific bulk populations were characterized by evaluating: 1) lysis on panels of targets from normal donors; 2) inhibition of lysis by anti-class II monoclonal antibodies; and 3) lysis on panels of HLA-mutant lymphoblastoid cell lines (LCL). Data from the first two approaches indicated: 1) that all CTL clones were DPw2-specific; and 2) no differences in fine specificity. However, lysis of different LCL mutants revealed complexities. Some of the CTL clones, which we designate "orthodox", showed the expected pattern of lysis of mutant LCL by lysing all targets which expressed DPw2. However, other CTL clones failed to lyse some of the mutant LCL that express DPw2; we designate these clones "heterodox". The heterodox clones identify differences

among serologically indistinguishable $DR^-/DPw2^+$ mutants. Cold target blocking experiments with the mutant LCL confirm differences in: 1) specificity among CTL clones and 2) CTL-defined phenotype among serologically indistinguishable DR^- $DPw2^+$ mutant LCL. RNA blot analysis of four such mutant LCL demonstrated perfect correlation between susceptibility to killing and expression of DRα. These data suggest that DRα contributes to recognition by some "DPw2-specific" CTL clones, perhaps because they recognize: a) DRα in a DPw2-restricted fashion, or b) a heterodimer formed by DRα and DPw2β.

The second general line of investigation relates to generation of HLA-mutant cell lines which have been (negatively) selected for resistance to lysis by one of the DPw2-specific CTL clones mentioned in the previous paragraph. The parental lines used for the generation of these mutants are diploid LCL from the DeMars collection described above, however the critical difference is that the mutants have been directly (negatively) selected by T cell recognition rather than for loss of a serological marker. This approach was undertaken in the belief that such mutants would provide information which would complement the findings with serologically selected mutants and perhaps provide surprising new insights. Mutants have been successfully isolated using several different protocols: A) 4 HLA diploid mutant lines have been isolated following ICR191-induced mutagenesis of an HLA-diploid (DPw2, DPw4) cell line; and B) more than 20 HLA-haploid mutant lines have been isolated following ICR191 and gamma-ray induced mutagenesis of an HLA-haploid (DPw2) cell line. All such mutants are completely resistant to lysis by the clone used for negative selection, as well as by all other DPw2-specific clones and bulk populations tested. However, the functional defect appears restricted to DPw2 recognition, since lysis of these targets is normal for CTL of other specificity. Among these mutants, residual DPw2-expression (detected by a monoclonal antibody ILR1) is observed only in a subset of the diploid lines (EM1, EM2, and EM5). The molecular genetic analysis of the lines derived from diploid parental has been complicated by the presence of two DP alleles. However, evaluation of DPβ expression by a very sensitive RNAase protection assay demonstrates that DPw2β expression is lost only in the diploid mutant which has completely lost DPw2 expression; the other three mutants (EM1, EM2, and EM5) retain DPw2β expression. Current studies are directed at testing the hypothesis that the defect in EM1, EM2 and EM5 results from lost expression of the DPw2α. If so, our results establish the important finding that the limited polymorphism of the DPα chain (between DPw2 and DPw4) is nevertheless critical for T cell recognition.

The third line of investigation relates to studies of CTL recognition of DPw2 gene products when DP genes are transfected into DPw2-negative cells. As outlined in last years annual report, such studies have generally failed to demonstrate T cell recognition. Recent studies have also failed to demonstrate normal recognition of the DP gene products even when transfected in HeLa cells. Such studies are always complicated by the fact that expression of DPw2 is generally lower in the transfected cells than in typical $DPw2^+$ LCL. One agent, butyrate, which induces increased expression of the transfected DP genes in L cells, also allows detectable DPw2-specific lysis of the transfected cells. The possibility was explored that this might reflect butyrate induction of other processes, such as enhanced capacity for conjugate formation, but no evidence was found to support that hypothesis.

Significance to Biomedical Research and the Program of the Institute: Because genes of the HLA region are crucial in controlling immune responses, in transplantation, and increasing the risk of a large variety of diseases, therapeutic intervention related to these phenomenon will likely depend on further understanding of the genes in this region, and their recognition by T cells. The DP subregion of HLA defined previously in this project promises to be an informative one; the present studies clearly indicate that the way T cells "see" DP is rather more complex than one might have predicted. Since such details of T cell recognition are essential to normal and pathophysiological host responses, our understanding of them may be essential to unravel the events involved in tumor recognition by T cells and in autoimmune responses.

Proposed Course of Project: We will continue to characterize the genetics, structure and function of products of the DP region of the HLA complex. Our emphasis will continue to be on the functional characterization of the DP products by T cell recognition. A major emphasis will be generation and characterization of further mutant LCL which have been selected for resistance to lysis by DPw2-specific CTL clones. Previously generated mutants will be more fully characterized to establish the molecular basis for the observed phenotypic defects; we expect such studies will establish the critical role of DPα gene polymorphism in T cell recognition of DP. Further mutants will be generated using "point" mutagens and an HLA-DPw2 haploid parental cell in order to maximize yield of mutant cells with limited alterations in primary sequence. Structural characterization of these mutants, in collaboration with Dr. E. Cowan, is expected to complement other approaches to understanding details of the structure function relationship between class II structure and T cell recognition.

Publications:

Cohen, N., Shaw, S., Amar, A., Oksenberg, J., and Braggart, C. 1984. HLA-linked SB antigens in Israel: Population study, analysis of homozygous typing cells and generation of local SB reagents. Tissue Antigens 24:113-120.

Hartzman, R., Shaw, S., and Robbins, F. M. 1984. Origin and expansion of SB (DP)-specific reagents characterized in the 9th International Workshop. In Histocompatibility Testing 1984. Mayr, M., E. Albert, and M. P. Baur, eds. Springer-Verlag, Berlin, pp 303-305.

Morling, N., Hartzman, R. J., Jakobsen, B. K., Oedum, N., Platz, P., Ryder, L. P., Shaw, S., and Svejgaard, A. 1984. Typing for DP-like determinants. In Histocompatibility testing 1984. Mayr, M., Albert, E., and Baur, M. P., eds. Springer-Verlag, Berlin, pp 469-469.

Sanchez-Perez, M., and Shaw, S. 1985. HLA-DP: Current Status. In Human class II histocompatibility antigens. Theoretical and practical aspects--clinical relevance. Ferrone, S., Solheim, B. G., and Moller, E. eds. Springer-Verlag, New York. In press 1985.

Shaw, S. 1985. Non-immunological functions of MHC and MHC-linked genes. ASHI Quarterly 9:12-12.

Shimizu, Y., Koller, B., Geraghty, D., Orr, H., Shaw, S., Kavathas, P., and DeMars, R. 1986. Transfer of cloned human class I MHC genes into HLA-mutant human lymphoblastoid cells. Mol. Cell. Biol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05103-05 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure and Function of Cytotoxic T Lymphocyte Granules

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

PI:	P. A. Henkart	Senior Investigator	IB, NCI
Others:	T. Soares	Microbiologist	IB, NCI
	W. Munger	Staff Fellow	IB, NCI
	J. Bluestone	Laboratory Leader	IB, NCI
	C. Yue	Medical Staff Fellow	IB, NCI
	R. Hodes	Senior Investigator	IB, NCI
	M. Taplits	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

M. V. Sitkovsky	Senior Staff Fellow	LI, NAI

LAB/BRANCH
Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Cytoplasmic granules of cytotoxic T lymphocytes have been studied in order to assess their role in the lytic function of these cells. Granules from cloned CTL have been purified by Percoll gradient centrifugation and shown to contain two serine esterases capable of cleaving the trypsin substrate BLT in addition to the lytic cytolysin protein and a nuclear DNA releasing activity. The major BLT esterase in CTL is a 60kd protein, comprised of two 30kd protein chains linked by disulfide bonds. The enzyme is inactivated by diispropylfluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), but these reagents react with the enzyme in intact cells inefficiently. This was shown to be caused by a low internal pH of the intact granules inside the cells, since agents known to raise the pH of acidic organelles (ammonia, chloroquine, monensin and nigericin) acted synergistically with PMSF in the inactivation of BLT esterase in intact cells but not the solubilized enzyme. The same selective synergistic effect was observed in the labeling of this protein band with radiolabelled DFP. When lytic function of CTL whose BLT esterase was greater than 95% inactivated by these treatments was examined, it was found to be virtually unaffected, indicating that the BLT esterase is probably not required for the CTL lytic process. The secretion of CTL BLT esterase into the medium has been shown to be a convenient marker for the granule exocytosis process. Using this assay, we have studied in vivo generated CTL from peritoneal exudates in which allo tumors have been recently rejected. Two directional allo-specific BLT esterase secretion has been demonstrated from such CTL, confirming the granule exocytosis mechanism operates in these cells during the lytic process. In addition to these studies with classical CTL, we have found that cloned helper T lymphocytes contain cytoplasmic granules with high levels of BLT esterase. At least some of these helper clones are capable of potent lytic function. We have found that BLT esterase is secreted into the medium by specific antigenic and MHC-restricted stimuli.

Project Description

Objectives: To characterize the cytoplasmic granules of cytotoxic T lymphocytes and other cytotoxic lymphocytes and ascertain their role in the lytic function.

Methods employed: Cloned alloreactive cytotoxic T cells are grown in in vitro culture with IL2 from EL4 and ConA stimulated spleen cell supernatents. They are cloned by limiting dilution methods and assayed for cytotoxicity by the ^{51}Cr release method. Granules were purified by the previously described Percoll gradient method. Cytolysin activity was measured routinely by serially diluting the material in PBS and adding an equal volume of SRBC suspended in BSS. Hemolysis was measured by hemoglobin release. ^{51}Cr release was also used with nucleated cell targets. BLT (α-Cbz-lysine-thiobenzyl ester) hydrolysis was measured by a spectrophotometeric assay using Ellman's reagent.

Major Findings: Cytoplasmic granules of cytotoxic T lymphocytes have been studied in order to assess their role in the lytic function of these cells. Granules from cloned CTL have been purified by Percoll gradient centrifugation and shown to contain two serine esterases capable of cleaving the trypsin substrate BLT in addition to the lytic cytolysin protein and a nuclear DNA releasing activity. The major BLT esterase in CTL is a 60kd protein, comprised of two 30kd protein chains linked by disulfide bonds. The enzyme is inactivated by diispropylfluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), but these reagents react with the enzyme in intact cells inefficiently. This was shown to be caused by a low internal pH of the intact granules inside the cells, since agents known to raise the pH of acidic organelles (ammonia, chloroquine, monensin and nigericin) acted synergistically with PMSF in the inactivation of BLT esterase in intact cells but not the solubilized enzyme. The same selective synergistic effect was observed in the labeling of this protein band with radiolabelled DFP. When lytic function of CTL whose BLT esterase was greater than 95% inactivated by these treatments was examined, it was found to be virtually unaffected, indicating that the BLT esterase is probably not required for the CTL lytic process. The secretion of CTL BLT esterase into the medium has been shown to be a convenient marker for the granule exocytosis process. Using this assay, we have studied in vivo generated CTL from peritoneal exudates in which allo tumors have been recently rejected. Two directional allo-specific BLT esterase secretion has been demonstrated from such CTL, confirming the granule exocytosis mechanism operates in these cells during the lytic process. In addition to these studies with classical CTL, we have found that cloned helper T lymphocytes contain cytoplasmic granules with high levels of BLT esterase. At least some of these helper clones are capable of potent lytic function. We have found that BLT esterase is secreted into the medium by specific antigenic and MHC-restricted stimuli. In general, these stimuli parallel closely those required for proliferation of these clones; however, IL-2 does not cause measurable secretion while generating a stong proliferative responsel Thus BLT esterase secretion provides a new insight into helper T cell physiology.

Significance to Biomedical Research and the Program of the Institute: It is clear that the cell-mediated immune system is responsible for many fundamental properties of the body's overall defense system against foreign organisms. This also appears to be true of the body's natural defenses against tumors. We are bringing a new means of analysis to bear on the cells which mediate these activities so that they can be understood on a molecular basis. This knowledge should be of great benefit in designing new therapeutic modalities.

Proposed Course of Project: We will coordinate studies of CTL granules with ongoing biochemical studies of LGL granules to ascertain if other proteases are present CTL granules. Our primary focus is to define the physiology of granule exocytosis in these cells since the BLT esterase provides a completely new opportunity to study this process. Various non-cellular stimuli including monoclonal antibodies against the T cell receptor will be tested in order to define precisely the requirements for secretion. In the case of the helper T cell clones, we will try to ascertain if the cytoplasmic granules containing BLT esterase are related to the cytotoxic capacity of these cells or if they may be also important for helper functions.

Publications:

Henkart, P., Henkart, M., Millard, P., Fredrikse, P., Bluestone, J., Blumenthal, R., Yue, C., Reynolds, C. W.: 1985. The role of cytoplasmic granules in cytotoxicity by large granular lymphocytes and cytotoxic T lymphocytes. Adv. Exp. Med. Biol. 184:121-134.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05104-05 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Detection and Analysis of H-2 Variant Cell Lines from Murine T Cell Lymphomas

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

PI: G. M. Shearer Senior Investigator IB, NCI

COOPERATING UNITS *(if any)*

T. H. Hansen, Department of Genetics, Washington University, St. Louis, MO

LAB/BRANCH

Immunology Branch

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Various lines of the S-49 T cell lymphoma of BALB/c origin are being studied for the expression of H-2 antigens. Normal BALB/c lymphocytes express H-2K^d, H-2D^d, and H-2D^d antigens. We have found that the five lines of the S-49 lymphoma thusfar studied do not express all of these cell surface H-2 antigens. The patterns of expression of H-2 antigens using these cells as targets for: (a) antibody and complement; and (b) cytotoxic T lymphocytes (CTL) exhibit four different patterns of H-2^d expression in the five lines tested. This system may be of value for investigating regulation of expression of major histocompatibility complex (MHC) antigens, and raises the possibility of a relatively high rate of modulation of these antigens among tumor cell lines of the same origin.

No progress was made in this project during the past year.

Project Description

Objectives: The purpose of this project is to study the expression of H-2 antigens on different lines of a T cell lymphoma, to determine the mechanism(s) of differential expression of these antigens on the cell lines and to establish whether we can detect changes occurring in H-2 expression as we carry these lines in vitro and in vivo.

Methods Employed: The various tumor lines as well as "wild type" BALB/c cells were tested for expression of K^d, D^d and L^d antigens by CTL, monoclonal reagents plus complement, and monoclonal reagents using FACS. Internal labelling using "^{14}C-leucene for immunoprecipitation studies were performed to determine whether the lack of expression of these H-2 antigens are a problem of cell surface antigen expression or intra-cellular synthesis.

Major Findings: Of the five lines of S-49 thusfar examined, at least four different patterns of H-2^d antigen expression has been observed: the "7.3" and "TAS" lines (actually splits of the same line) $K^d(-)$, $D^d(+)$, $L^d(-)$; "100.0" $K^d(-)$, $D^d(-)$, $L^d(-)$; S-49.1 $K^d(+)$, $D^d(+)$, $L^d(-)$; S-49-Thy-$K^d(+)$, $D^d(-,,L^d(-)$. Using BALB/c anti-pool sera as well as BALB/c anti-pool CTL, both of which should detect antigens of any other known H-2 haplotype, no other H-2 antigens have been detected. This is compatible with the hypothesis that these cell lines are not mixed up or contaminated with any other murine tumor cell lines that would express other H-2 antigens. Due to projects of a higher priority, little progress has been made on this project during the past year.

Significance to Biomedical Research and the Program of the Institute: The modulation of major histocompatibility complex (MHC) antigens by tumor cells provides an interesting model for investigating expression of these antigens on cell surfaces as well as gene expression. Furthermore, since autologous tumor antigens appear to be recognized in association with syngeneic MHC antigens by T lymphocytes, the modulation of MHC antigens may be a mechanism by which tumors could escape rejection. The tumor lines we have identified that have "lost" certain H-2 antigens could represent lines which have been selected to grow in host mice by such an "escape" mechanism.

Proposed Course of Project: Biochemical analysis at the levels of cell surface expression and intracellular synthesis will be performed in order to understand this antigenic modulation. Other S-49 cell lines will be studied in an attempt to identify additional lines which exhibit differential H-2^d antigen expression. An extensive panel of anti-H-2^d monoclonal reagents will be employed to elucidate the fine specificity of the antigens expressed by these tumor cell lines. The tumor lines will be injected into syngeneic BALB/c and H-2 allogeneic mice in an attempt to recover cell lines that exhibit additional differences in H-2^d antigen expression. If a number of the above experiments are interesting it will be important to determine if the phenomenology is more general, and could be demonstrated for other murine T cell lympomas. Attempts will be made to induce expression of negative H-2^d antigens by culture with gamma interferon and other agents that increase the amount of class I MHC antigens expressed. Regulation of failure to express these H-2^d antigens will be investigated at the molecular level.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05106-05 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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	IB, NCI
Others:	R. Abe	Visiting Fellow	IB, NCI
	B. Needleman	Medical Staff Fellow	IB, NCI
	J. Bluestone	Lab Leader	IB, NCI
	D. Singer	Senior Investigator	IB, NCI
	M. Pescovitz	Senior Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

Department of Pathology, University of Utah, Salt Lake City, UT

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	1.0	

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☐ (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 alloreactive T cell repertoire has been analyzed for the influence of non-MHC determinants on alloreactivity. The responses of cloned T cells to Mls^a were analyzed in clones generated specifically to Mls^a stimulator cells as well as in clones specific for soluble antigens (antigen specific), foreign I region products (alloreactive), or syngeneic I products (autoreactive) which showed cross reactive recognition of Mls^a. It was demonstrated through the use of congenic strains, recombinant inbred strains, and monoclonal antibodies, that cloned T cell responses were specific for Mls and that this recognition was MHC restricted. The overall importance of non-MHC encoded determinants in alloreactive T cell recognition was surveyed by examining a panel of 70 antigen specific and/or autoreactive T cell clones for coincidental alloreactivity. Approximately 80% of the clones analyzed revealed one or more alloantigen specific cross reactivities. In several of these instances, specificity appeared to be for MHC products alone, without detectable influence of non-MHC genes. However, in the majority of instances, alloreactive specificity involved non-MHC as well as MHC gene products expressed by stimulating populations. These findings therefore suggest that the high frequency of alloreactive T cells observed in multiple experimental systems may reflect a frequent role of non-MHC as well as MHC encoded gene products.

The cytotoxic T cell repertoire specific for class I allogeneic and xenogeneic determinants was studied. Through the use of radiation bone marrow chimeras, it was demonstrated that responsiveness to K^b mutant determinants was the outcome of unique interactions between both T cell genotype and maturation environment. Through the use of a transgenic mouse model, in which porcine class I genes had been introduced into the germ line of murine cells, it was demonstrated that normal murine T cells expressed a cytotoxic T cell repertoire specific for xenogeneic class I determinants expressed on mouse cells. This repertoire was cross reactive

Project Description

Objectives: T cell responses to Mls or MHC encoded alloantigens appear to be unique in the strength of these primary responses and in the correspondingly high precursor frequency of responding T lymphocytes. For both these conceptual reasons, and because of the importance of these loci in transplantation biology, a more complete understanding of response mechanisms to these determinants is both interesting and important.

Methods Employed: Mls-specific T cell clones were generated by in vitro culture and limiting dilution techniques, employing stimulator populations expressing Mls^a determinants. Other clones were generated in similar fashion by repeated stimulation with appropriate soluble antigens and presenting cells.

Studies of responses to the mutant K^b products employed a series of mutants including K^{bm1} and the K^{bm6}. Responses to these determinants were studied in assays of CML. Responding populations included normal spleen cell populations, radiation bone marrow chimeric spleen cell populations, and spleen cells from neonatally tolerized animals. Isolation of the swine class I MHC gene PD1 and its introduction by microinjection into fertilized mouse embryos are described in detail in Project No. Z01CB05124-01 I. Cytotoxic T cell responses specific for transgenic MHC products were evaluated through conventional techniques of in vitro sensitization and cytotoxic T cell assays.

Major Findings:

1) Cloned T cells were generated which are specific for Mls^a determinants, as demonstrated by strain distribution and by responses to recombinant inbred lines derived from (B6 x DBA/2)F_1 mice (heterozygous for Mls^a). The use of H-2 congenic and F_1 stimulating cells demonstrated that Mls^a was recognized by most clones in the context of $H\text{-}2^b$, $H\text{-}2^d$, or $H\text{-}2^k$, but not $H\text{-}2^q$ products. Individual clones utilized I-A or I-E products as restricting elements. A number of other T cell clones which were generated to be specific for soluble antigens (antigen-specific), foreign I region products (alloreactive), or syngeneic I products (autoreactive) also showed cross-reactive recognition of Mls^a in a similarly H-2-restricted fashion. The MHC restriction specificity of Mls^a recognition by cloned T cells was confirmed through the use of monoclonal anti I-A and I-E antibodies. Recently, series of T cell clones specific for either Mls^a or Mls^c have been generated and their specificities confirmed. The use of these clones has permitted the demonstration that the Mls system as currently defined is polymorphic, i.e. Mls^a or Mls^c reactive clones show reciprocal specificity patterns for stimulatory cells of the appropriate allele. In addition, back cross analysis is currently in progress to define the allelism or non-allelism of Mls^a and Mls^c.

2) B6, B10, or B10.D2 mice, as well as B10.D2 --> B10 chimeric cells were all able to generate strong CML responses to K^b mutant bm1 and bm6 determinants. In contrast, however, B10 --> B10.D2 chimeric T cells had a markedly reduced response to bm1 and were entirely unresponsive to bm6, in spite of their normal alloreactivity to third party B10.BR. The same selective unresponsiveness to bm6 was observed in T cells from B6 --> bm1 chimeras, indicating that K region differences in maturation environment were sufficient to cause this effect.

These findings demonstrate that the alloreactive T cell repertoire to these mutant K region determinants is not the product of T cell genotype alone (since either H-2^b or H-2^d T cells can respond) or of the T cell maturation environment alone (since T cells which have matured in either an H-2^b or H-2^d environment can be responsive). Rather, the alloreactive T cell repertoire appears to be the unique outcome of interaction between T cell genotype and maturation environment, paralleling the phenomenon previously demonstrated for MHC-restricted T cell recognition of conventional antigens.

3) Normal B10 T cells were capable of generating cytotoxic T cell responses specific for transgenic B10 mice which expressed the serologically detectable product of the xenogeneic PD1 class I MHC gene. The specificity of this cytotoxicity was confirmed employing transfected L cell targets and by inhibition with alloantisera. In order to analyze the T cell repertoire specific for the transgenic gene product, cytotoxic T cells generated by a B10 antitransgenic sensitization were assayed for cross reactivity against a panel of allogeneic murine target cells. It was observed that there was variable cross reactive lysis on a panel of fully MHC allogeneic target cells. In contrast, a consistent and strikingly high degree of cross reactivity was seen against a number of K^b mutant target cells. These findings suggest that the expressed cytotoxic T cell repertoire for xenogeneic class I MHC products is cross reactive with allogeneic class I determinants and that this repertoire may bear a selective relationship to the K^b mutant specific repertoire.

4) In order to assess the nature of T cells active in allograft rejection, a number of T cell clones was evaluated for their ability to mediate graft rejection in vivo. Both Ia specific and Mls^a specific T cell clones were competent in mediating graft rejection by nude mice. The comparative specificities expressed by these clones in vitro and in vivo is currently under study.

Significance to Biomedical Research and the Program of the Institute: It remains a central question whether the mechanisms of immune repertoire generation are identical for conventional antigens and for MHC encoded antigens. In this respect, the results of the current studies have demonstrated that in several respects the strong T cell responses to both Mls and mutant K^b determinants parallel response mechanisms studied for MHC-restricted responses to conventional antigens. The most immediately apparent implications of such findings include their application to situations such as clinical bone marrow transplantation, in which complex influences can be anticipated upon the ultimate host response repertoire to transplantation as well as conventional antigenic challenge. Analysis of the T cell repertoire toward xenogeneic transplantation antigens will bear specific relevance for the facilitation of xenogeneic tissue or organ grafting.

Proposed Course of Research: Antibodies specific for T cell receptor structures will be utilized to determine whether the receptors used in recognition of Mls^a are the same or different from those used in recognition of other specificities. 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.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05107-05 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

T Cell Responses to Minor Histocompatibility Antigens

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

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Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The ability to generate cytotoxic T lymphocyte responses to minor H antigens offers a potent tool for the study of self-tolerance and self-recognition. Results obtained in this system have thus far demonstrated 1) that the self + X T cell repetoire is highly cross-reactive for allogeneic MHC determinents suggesting that the response to allogenic MHC antigens is comprised of multiple self + X specificities and 2) that self minor H determinents tolerize T cells only in association with self MHC determinents so that tolenance induction to non MHC self components is restricted by MHC encoded products. We are currently examining the role of antigen processing in the generation of the cytotoxic response to minor-H antigens. Results so far indicate that 1) Macrophages are requisite for minor H specific CTL generation in vitro. 2) The minor antigens need not be synthesized by the antigen presenting cell but can be acquired in vitro by macrophages and subsequently presented in an immunogenic fashion. 3) The generation of the CTL by non antigen bearing APC's is inhibitable by antibodies to the T4 molecule expressed by Ia-restricted T helper cells.

Project Description

Objective: These experiments will study the mechanism of the development of tolerance and reactivity to minor histocompatibility antigens.

Methods Employed: Normal and chimeric mice are primed with spleen cells which express allogeneic minor H antigens. After 2.5 weeks, recipient thymus or spleen cells are restimulated in vitro and the generation of cytotoxic T cells specific for allogeneic minor H antigens is assessed. Spleen cells from mice primed in vivo to minor H antigens were either left unseparated or were macrophage depleted. Depletion was accomplished via G10 Sephadex columns. Purified accessory cell populations (obtained via RAMB/complement treatment of whole spleen population) of either stimulator or responder phenotype were added and were both shown capable of restoring the response. The effects of MT4, L3T4 and anti IA antibodies are being assayed in an effort to establish the dependency or independence of the response on IA molecules. The effects of chloroquin, a drug capable of interfering with antigen processing are also being studied in an effort to delineate the role of processing.

Major Findings: 1) The MHC dependence of T cell tolerance induction to minor H antigens was investigated using parent --> F_1 radiation bone marrow chimeras of the form C3H.SW --> (B10xBR)F_1. In these chimeras, the donor and host differ by the minor H antigens associated with the C3H and Black strains, share the H-2^b MHC and the host bears additionally the H-2^k MHC. These chimeras were found to be specifically tolerant to the minor C3H antigens of the donor in the MHC context of donor H-2^b determinants. However the chimera was reactive to the donor minor H antigen in the context of the non-donor MHC (i.e., they were reactive to C3H [H-2^k]). Hence the tolerance pattern observed in this animal demonstrates that T cells are not tolerant to self minor H antigens alone but are only tolerant to these antigens in the MHC context in which they appear in the animal. 2) Macrophages are requisite for minor H specific CTL generation in vitro. 3) The minor antigens can be acquired, processed, and presented by macrophages. 4) The generation of minor H specific CTL by APC is inhibitable by the MT4 antibody.

Significance to Biomedical Research and the Program of the Institute: Insights into the mechanism of self-tolerance are important to our understanding of the function of the immune system. As the underlying principles become clarified, it is anticipated that they will have significant impact on human transplantation and the immunological approach to cancer treatment.

Proposed Course of the Project: To further elucidate the rate of antigen processing in the generation of CTL reactivity and tolerance to minor H antigens.

Publications: None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05108-04 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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	IB, NCI
Others:	M. Taplits	Medical Staff Fellow	IB, NCI
	A. Finnegan	Investigator	IB, NCI
	K. Hathcock	Chemist	IB, NCI
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COOPERATING UNITS *(if any)*

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☐ (a1) Minors

☐ (a2) Interviews

B

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

It was previously demonstrated that B cell responses mediated by antigen specific T helper cells were regulated by T suppressor cells through two distinct MHC restricted pathways. It was subsequently demonstrated that cloned lines of Lyt 1^+ 2^- $L3T4^+$ antigen specific and MHC restricted suppressor cells could also mediate suppressor effector function in these T dependent antibody responses.

Autoreactive T cell clones, specific for syngeneic I-A or I-E products were shown to function as T helper cells through two distinct pathways: One pathway was polyclonal and MHC unrestricted at the level of T helper-B cell interaction and the other was MHC restricted and dependent upon antigen specific triggering of responding B cells. MHC restricted, antigen-nonspecific suppressor populations which function to regulate responses by carrier specific T helper cells also function to regulate the responses mediated by autoreactive T helper cells. Activation of B cells by antigen specific and autoreactive T helper cells therefore appears to share susceptibility to similar regulatory influences.

The role of T cells in regulating the fine specificity of B cell antibody responses was studied by examining the T15 idiotype dominant response to phosphocholine (PC) 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.

Project Description

Objectives: This project is designed to evaluate the mechanisms of regulatory T cells controlling B cell activation and antibody response.

Methods Employed: The response systems studied are T cell and accessory cell dependent antibody response in vitro. Heterogeneous T suppressor populations are generated by in vivo carrier priming of mice, followed by in vitro culturing of T cells with accessory cells and appropriate antigen.

The generation of monoclonal T cell populations was accomplished through repeated in vitro stimulation of in vivo primed T cells in the presence of antigen presenting cells, antigen, and a source of T cell growth factor. Cloning was accomplished through limiting dilution techniques, and cloned cells propogated through repeated in vitro stimulation. Antibody and idiotype responses to PC were studied by in vitro responses of heterogeneous or cloned T helper cells and unprimed B cells. Responses were assayed either by enzyme-linked immunosorbant assays (ELISA) or by the measurement of plaque forming cells (PFC).

Major Findings:

1. Suppressor T Cells.

The activation of T suppressor (T_S) cells requires in vivo priming, followed by in vitro re-exposure of T cells to the specific priming antigen in the presence of appropriate antigen presenting cells. Two apparently distinct suppressor pathways are activated by this procedure: 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. Suppression occurred only when the determinants recognized by the cloned T suppressor cell, the determinant recognized by the cloned T helper cell, and the hapten recognized by the responding B cell were all covalently linked to one another. It thus appeared that a tripartite antigen mediated interaction of these three cell populations was essential for suppression of B cell responses.

2. Autoreactive T Cells.

A series of T cell clones was generated by limiting dilution which were specific in their proliferative responses for syngeneic I-A or I-E products. Some of these autoreactive T cell clones functioned as T_H cells for B cell responses. The same cloned T_H cells could function through two different pathways. In one pathway, a predominantly IgM response was induced in unprimed B cells; this response was polyclonal and was MHC unrestricted at the level of T_H cell-B cell interaction. In the second pathway, an IgG response was induced in primed B cells only in the presence of the specific priming antigen and via an MHC-restricted T_H-B cell interaction. Heterogeneous populations of Lyt 1^+2^- T suppressor cells, generated as outlined above, were also found to suppress the B cell responses mediated by autoreactive T helper cells. This suppression was mediated in an MHC restricted fashion similar to that observed in the regulation of responses by carry specific T helper cells.

3. Augmenting T (T_A) cells.

Titrations of cloned T_H cells defined conditions for the generation of optimal B cell responses supported by these T_H cells. It was found that B cell responses could be enhanced or augmented above these levels by the addition of unprimed Lyt 1^+2^- T cells. These T_A cells were MHC-restricted in their function and augmented responses only when their MHC restriction specificity matched that of the cloned T_H cells functioning in a given response.

4. Idiotype regulation.

Both heterogeneous and cloned KLH specific T helper populations were capable of eliciting PC specific antibody responses from unprimed B cells in response to the antigen PC-KLH. The responses generated by cloned and heterogeneous T helper cells were comparable in magnitude, isotype (predominantly IgM) and affinity. In addition, the proportion of T15 idiotype positive antibodies generated were comparably high in response to either heterogeneous or cloned T helper cells. Similar findings were observed when antibody responses were assayed by either ELISA or PFC assays. Cloned carrier specific and MHC restricted T cells were therefore sufficient to generate optimally T15 idiotype dominant responses from unprimed B cells. 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. Targeting of T helper cell function.

The ability to redirect T helper cell specificity was evaluated using antibody heteroaggregates consisting of an antibody to allotypic determinants on the T cell receptor (F23.1) covalently linked to antibodies directed at B cell surface determinants. The intent of these experiments was to determine whether T cell receptor specificity could be "redirected" and to identify those "receptor" determinants on the B cell surface which could serve as targets for T cell helper function. Preliminary findings have suggested that T cell help

can be targeted through this procedure toward Ia determinants on the surface of responding B cells.

Significance to Biomedical Research and the Program of the Institute: The immune response to both experimental antigens and to naturally occuring antigens of infectious agents or of tumors is determined in large part by the regulatory influences of helper and suppressor T cell populations. It has been possible through the studies outlined above to analyze the regulatory effects on antibody responses in vitro. This has in turn permitted the analysis of T cell subpopulation interactions, and most recently the identification of monoclonal regulatory cells. It is anticipatated that through the further study of such populations, cellular as well as molecular analysis of immune response regulation can be accomplished. It is further intended that such principles will be applied to models of immune response to tumor antigens. The demonstrated function of self-reactive T cells raises clear questions about the relevance of such cells in vivo. The regulation of these cells in normal and pathologic autoimmune states is now a question of biologic importance.

Proposed Course of Project: Current efforts are being directed at an analysis at the structural level of those determinants involved in regulatory cell interactions. In particular, the interaction between T cell receptor structures and target determinants on B cells and accessory cells is being analyzed.

Publications:

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Hathcock, K. S., Gurish, M. F., Nisonoff, A., Conger, J. D. and Hodes, R. J.: 1986. Influence of helper T cells on the expression of an intrastrain cross-reactive idiotype. Proc. Natl. Acad. Sci. 83:155-159.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05110-04 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Immune Studies in Homosexual Men at Risk for Acquired Immune Deficiency Syndrome

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

PI:	G. M. Shearer	Senior Investigator	IB, NCI
	H. B. Dickler	Senior Investigator	IB, NCI
Others:	R. C. Gallo	Chief	LTCB, NCI
	R. Yarchoan	Senior Investigator	COP, NCI
	S. Broder	Director	COP, NCI

COOPERATING UNITS *(if any)*

K. S. Tung, Department of Pathology, University of New Mexico, Albuquerque, NM
R. Schuloff, Dept of Immunology, George Washington University, Washington, DC
R. Redfield, Walter Reed Army Medical Center, Washington, DC

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [x] (b) Human tissues
- [ ] (c) Neither

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 proliferative (^{3}H) and cell-mediated lympholysis (CML) to influenza virus (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.

Using the above approach, we have tested T cell functions in AIDS patients undergoing therapy with the thymidine analog AZT. Some patients undergoing AZT exhibited a restoration of T cell immune function.

PBL from antibody (-) donors were infected with HTLV-III after stimulation with PHA or HLA alloantigens. PHA stimulated cells produced virus early but the cultures died within 2 weeks. In contrast, alloantigen stimulated cultures survived and produced virus for at least 40 days.

Project Description

Objectives: The purpose of this project is to investigate various immune parameters of individuals in the developmental stages of AIDS. The immune parameters of individuals are being followed for at least six years to determine the functional immune changes that accompany AIDS development.

Methods Employed: Peripheral blood leukocytes were separated on Ficoll-Hypaque and sensitized in vitro to influenza self and HLA alloantigens. Proliferative and cytotoxic T lymphocyte (CTL) assays were run under optimal conditions. Suboptimal sensitization conditions were also tested by sensitizing with lower concentrations of influenza virus. CD3, CD4 and CD8 analyses were run on the PBL using flow microfluorometry. Sera were tested for HTLV-III antibodies. PBL from donors were also tested for retrovirus activity by reverse transcriptase activity, and sera were tested for antibodies to HTLV-III. Cultures of PBL were infected with HTLV-III after stimulation with PHA or alloantigens.

Major Findings: Peripheral blood leukocytes (PBL) from approximately 50% of HTLV-III/LAV seropositive donors without symptoms of AIDS exhibited a selective absence of cytotoxic T lymphocyte (CTL) and proliferative responses to influenza virus (S+X), but not to HLA alloantigens (ALLO). The other 50% respond to both stimuli. More than 90% of patients with LAS or ARC exhibited such a selective absence of S+X T cell responses. All patients with AIDS failed to respond to S+X and 50% also failed to respond to ALLO. A sequential loss of S+X and a subsequent loss of ALLO was observed in a limited number of donors who progressed from seronegative to seropositive and on to symptomatic AIDS. The S+X responses of donors who exhibited the selective loss could be reconstituted in vitro by co-stimulation of the PBL with influenza virus plus interleukin 2 or HLA alloantigens.

Analysis of the selective loss of S+X in seropositive donors was tested using PBL from seronegative donors by cell fractionation and monoclonal antibody and complement treatment. It was concluded that two distinct T helper cell pathways exist, one that is $CD4^+$ and the other that is $CD4^-$. the $CD4^+$ pathway appears to be required for self-restricted antigens, whereas allogeneic responses appear to be able to utilize either pathway. Thus, functional loss of T helper cell function is limited to S+X responses during the developmental stages of AIDS.

PBL from AIDS patients (from the Clinical Oncology Program) were tested in the functions outlined above before, during, and after treatment with AZT, a thymidine analog that has anti-retroviral activity. No patients responded to S+X before treatment, but approximately 30% exhibited normal responses to S+X during and after therapy. This change coincided with a resolution of the patients' Kaposi's lesions.

PBL from seronegative donors were infected with HTLV-III after stimulation with either PHA stimulus (commonly used for HTLV-LLL infection) or with HLA alloantigens. Infection occurred after both stimuli. Stimulation with PHA, large amounts of virus were produced for a brief period (~ 10 days) after which the cultures died. In contrast stimulation with alloantigens resulted in a lower and slower production of HTLV-III, but the cultures survived and continued to

produce virus for at least 40 days. We detected no appreciable differences whether the cultures were stimulated with class I and/or class II HLA antigens. Thus, the extent of HTLV-III infection and progression of AIDS may depend on activation of T cells by stimuli other than HTLV-III alone.

Significance to Biomedical Research and the Program of the Institute: Understanding the immunology associated with the development of AIDS is a high priority of the biomedical research community and particularly of the NCI. The evaluation of immune parameters of high risk groups may be predictive for AIDS development and may elucidate the mechanism(s) leading AIDS, and permit identification of susceptible individuals.

Proposed Course of Project: These donors will be followed for a period of up to two years with immune evaluation every three-to-six months. Individuals whose immune status appears to be of interest will be followed more extensively. Healthy homosexual donors who have antibodies to HTLV-III but no evidence for infection will be followed intensively for evidence for HTLV-III virus and changes in immune function. We shall more extensively investigate the phenomenon of enhanced infection of PBL cultures after stimulation with HLA alloantigens, and attempt to determine whether elevated allogeneic T cell immune activity is a cofactor for AIDS susceptibility. A more detailed study of the subpopulations of human T helper cells will be made, including the antigens which these subsets recognize. For example, we have recently found that human T cell recognition of murine xenoantigens requires the $CD4^+$ helper pathway. We shall also elucidate the down-regulatory effects of human allogeneic CTL responses, the removal of this down regulation by Sephadex G10 fractionation, and the observation that PBL from many HTLV-III seropositive individuals are not down-regulated in this way.

Publications:

Tung, K. S., Koster, F., Bernstein, D. C., Kriebel, P., Payne, S. M., and Shearer, G. M.: 1985. Elevated allogeneic cytotoxic T lymphocyte activity in peripheral blood leukocytes of homosexual men. J. Immunol. 135:3163-3171.

Shearer, G. M.: AIDS: An autoimmune pathologic model for the destruction of a subset of helper T lymphocytes. The Mount Sinai Journal of Medicine, in press.

Shearer, G. M., Salahuddin, S. Z., Markham, P. D., Joseph, L. J., Payne, S. M., Kriebel, P., Bernstein, D. C., Biddison, W. E., Sarngadharan, M. C. and Gallo, R. C.: 1985. Prospective study of cytotoxic T lymphocyte (CTL) responses to influenza and antibodies to HTLV-III in homosexual men: selective loss of an influenza-specific, HLA-restricted CTL response in HTLV-III positive individuals with symptoms of AIDS and in a patient with AIDS. J. Clin. Invest. 76:1699-1704.

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patients with AIDS or AIDS-related complex. The Lancet I:575-580.

Singer, A., and Shearer, G. M.: 1986. AIDS therapy by blocking $CD4^+$ cells. Nature (Scientific Correspondence) 320:113.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05111-04 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

PI:	S. McCarthy	Staff Fellow	IB, NCI
Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have analyzed the signals influencing the generation of MHC class II allospecific CTL and have found that the development of these CTL is actively regulated in primary in vitro cultures (MLC) by Lyt2$^+$ T cells triggered in response to MHC class I alloantigens. Class II allospecific CTL can be readily generated in primary cultures, but the presence of a simultaneous class I MHC stimulus in these cultures causes a marked reduction of class II-specific CTL activation. This reduction can be prevented by adding to culture a dose of monoclonal anti-Lyt2 antibody that does not block the generation of class I-specific CTL. The role of MHC class I alloantigens in the regulation of class II allospecific responses illustrates that T cells recognizing class I and class II MHC antigens in MLC interact in a complex, and non-reciprocal, manner to influence the final effector T cell repertoire elicited by this complex immunogenic challenge.

Project Description

Objectives: The primary objective of this project is to identify the cellular interactions regulating the generation of cytolytic T lymphocytes specific for class II MHC alloantigens.

Methods Employed: Primary in vitro CTL responses were induced in mixed leukocyte cultures of spleen cells from H-2 congenic mouse strains. CTL activity generated during the five-day culture was assayed by lysis of ^{51}Cr-labeled LPS-activated target cells, which express high levels of class II MHC surface antigens. CTL effector cells generated during culture were characterized by negative selection (with monoclonal antibody plus complement treatments) and by blocking of CTL function with monoclonal antibody in the absence of complement. Regulatory cells were characterized by negative selection and by functional blockade with monoclonal antibody in the five-day culture.

Major Findings: The generation of CTL specific for allogeneic MHC class II determinants is actively regulated during primary in vitro mixed leukocyte cultures stimulated with a full MHC (class I + II) antigenic disparity. $Lyt2^+$ regulatory cells, triggered by MHC class I alloantigens in these cultures, inhibit or "preclude" the development of CTL activity against class II alloantigens also present in the cultures. Both $Lyt2^+$ and $L3T4^+$ class II-specific precursor CTL are sensitive to this inhibition, while mature class II-specific effector CTL are resistant to preclusion by the regulatory cells. Thus, preclusion acts on the development, but not on the expression, of CTL activity. The action of class I-induced $Lyt2^+$ regulatory cells on class II-specific CTL development can be prevented, or circumvented, by anti-Lyt2 monoclonal antibody (in the absence of complement) in culture. Anti-Lyt2 antibody in culture may directly block regulatory cell alloantigen recognition and activation, or it may interfere with the action or reception of the regulatory signal directed at class II allospecific precursor CTL. Preliminary results indicate that anti-Lyt2 monoclonal antibody-mediated "rescue" of class II-specific CTL activity from preclusion may require an interaction pathway involving $L3T4^+$ as well as $Lyt2^+$ cells.

Significance to Biomedical Research and the Program of the Institute: Identification of the regulatory elements responsible for immunodominance in the generation of allospecific CTL is necessary in the development of strategies for increasing CTL activity toward malignancies and decreasing or averting CTL responses toward allogeneic tissue grafts.

Proposed Course of Project: We expect to extend these studies to other systems, particularly self MHC-restricted T cell responses, in which immunodominance has been reported but is only poorly understood at present. Analysis of the regulatory interactions operating in these adaptive immune responses may suggest protocols by which protective immunity can be optimally induced.

Publications:

Golding, H., Bluestone, J., Satz, M. L., Singer, D. S., and Singer, A.: Generation of primary murine CTL specific for allogeneic and xenogeneic MHC determinants upon stimulation with murine L cells transfected with class I genes. J. Immunol. 134:3557-3559, 1985.

Golding, H., McCluskey, J., Munitz, T. I., Germain, R. N., Margulies, D. H., and Singer, A.: T cell recognition of a chimaeric class II/class I MHC gene product and the role of L3T4. Nature 317:425-427, 1985.

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Golding, H., Mizuochi, T., and Singer, A.: Specificity, phenotype, and precursor frequency of cytolytic T lymphocytes specific for class II major histocompatibility antigens. J. Immunol. 135:1610-1615, 1985.

Golding, H., Munitz, T. I., and Singer, A.: Characterization of antigen-specific, Ia restricted, L3T4$^+$ cytolytic T lymphocytes and assessment of thymic influence on their self-specificity. J. Exp. Med. 162:943-961, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05112-04 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Analysis of Recognition Structures on T Cells

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

PI:	J. A. Bluestone	Laboratory Leader	IB, NCI
Others:	D. H. Sachs	Chief, Transplantation Biology Section	IB, NCI
	O. Leo	Visiting Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The recognition structures of T cells have been examined using anti-receptor antibodies and anti-T cell surface antigen specific antibodies prepared against cytotoxic T cell clones. Antisera produced in mice, rats, and hamsters against the CTL clones show specific reactivity and immunoprecipitation of receptor molecules on the cytotoxic T cells as well as other molecules which appear to be critical to T cell recognition. Present studies are designed to look at monoclonal antibodies prepared against the T cell receptor and other cell surface antigens. One mAb has been generated that specifically activates CTL clones by binding in a clonotypic fashion to the T cell receptor. This mAb, 83-7-2, precipitates a 90 kd heterodimeric glycoprotein similar to that observed for other anti-receptor antibodies. The activation of CTL by the mAb resulted in lysis of Fc-receptor targets that did not express the nominal alloantigen recognized by the clone. In addition, a series of other monoclonal antibodies which recognize an Ly6-linked molecule on the surface of the cytotoxic T cell clones has been generated. These monoclonal antibodies appear to subdivide class I specific CTL into two distinct populations and one such mAb, 143-4-2 can activate CTL clones and a subset of $Lyt2^+$ T cells. Current studies are underway to develop monoclonal antibodies against isotypic determinations on the variable regions of the T cell receptor. In addition, further studies will be designed to analyze the monoclonal antibodies which have been developed and to better define the structural determinants expressed on cytotoxic T cells which are involved in T cell activation.

Project Description

Objectives: T cell responses to alloantigens encoded by the major histocompatibility complex are extremely important in immune responses associated with organ transplantation. Antibodies against structures expressed on alloreactive T cells could be useful in examining the immune response of T cells against alloantigens and might be employed both in vivo and in vitro to modify transplantation reactions and understand the repertoire of alloantigen specific T cells.

Methods Employed: Anti-T cell antibodies have been generated against several CTL clones specific for class I, K^b, MHC antigens. Mouse, hamster, and rat anti-T cell antibodies were produced by immunizing repeatedly with 10^7 cytotoxic T cells on a weekly basis. Immune sera were examined on the immunizing clone for effects on CTL function as well as cell surface staining using the Fluorescence Activated Cell Sorter (FACSII). Immune sera were also examined for cross reactivity against monoclonal anti-H-2 antibodies directed at the same MHC class I antigens as that recognized by the CTL clones. The assay used is a binding site specific assay which examines the ability of test sera or monoclonal antibodies to inhibit the binding of anti-H-2 antibodies to H-2 bearing lymphocytes. The cloned CTL used in these studies were isolated from alloreactive mixed lymphocyte cultures. CTL were cloned by limiting dilution at 1 cell/well in the presence of stimulator cells and 10% T cell growth factor (IL-2), screened for cytolytic activity in a 4 hr ^{51}Cr-release assay and subcloned at 0.3 cells/well where appropriate. Fine specificity of the cloned CTL was examined using a panel of MHC congeneic mouse strains, MHC mutant mice, and immunoselected MHC mutant cell lines. Inhibition assays were performed by preincubating effector cells with the test reagent for 30 min prior to the ^{51}Cr release assay. CTL activation was determined by screening for cytolytic activity of the CTL clone on radiolabeled Fc-receptor-bearing irrelevant targets in the presence of the mAb. Activation of resting T cell was studied by culturing T cells with the mAb and phorbol ester and assaying proliferation at day 4.

Major Findings: Alloreactive CTL clones have been used to immunize mice to generate antibodies against a T cell receptor molecule and other molecules associated with T cell function. In some instances, absorption with other CTL clones resulted in a clonotype specific antiserum. Antibodies have been identified in this serum which specifically inhibit the function of a series of CTL clones of different specificities and clearly react with determinants that are generally found on a large population of CTL. Three monoclonal antibodies have been derived from immunized mice, namely 120-4-24, 120-3-7-2, and 128-12-17. These three mouse monoclonal antibodies have been shown to specifically inhibit CTL function on a variety of CTL clones of different specificities. Further analysis of these monoclonal antibodies showed that the determinants detected are not expressed on resting spleen cells but are expressed after in vitro long term stimulation with allo class I but not class II antigens. Therefore, the expression of these molecules is clearly linked to the class specificity of this CTL. Biochemical analysis of the antigens detected by the monoclonal antibodies demonstrated that unique proteins are precipitated by each of these monoclonal antibodies. In the case of one monoclonal antibody, 120-3-7-2, the immunoprecipitated protein appears to be

similar to a T200 molecule which has been previously described. However, the other two monoclonal antibodies appear to precipitate proteins that have not yet been described and must apparently be important in T cell recognition. Another class of monoclonal antibodies have also been derived which recognize a molecule which is genetically linked to the Ly6 locus and likely to react with the Ly6 molecule. This molecule is expressed on resting peripheral T cells but not thymocytes and on all class I specific CTL clones that have been analyzed. $L3T4^+$ class II specific proliferating and CTL clones do not express these markers. However, the $Ly2^+$ class II specific CTL clones do express Ly6. These monoclonal antibodies have been examined on bulk CTL populations and shown to be expressed on a subpopulation of alloreactive class I specific CTL. A mAb, 143-4-2 has been generated which reacts with the Ly6.2C molecule and activates class I-specific CTL clones and naive $Lyt2^+$ spleen cells. Finally, an anti-clonotypic T cell receptor mAb 83-7-2 has been identified that activates a CTL clone, bm10-37. Thus, both anti-FcR and anti-Ly6.2C can redirect CTL lysis of a target that does not bear the nominal alloantigen. Current studies are underway to define the relationship between Ly6 expression and the TcR, the mechanism of T cell activation and the in vivo effects of these mAbs.

Significance to Biomedical Research and the Program of the Institute: A central problem in Transplantation Biology is the identification of specific methods to alter immune responses to transplantation antigens and thus effect allograft rejection. Since T cells are thought to be responsible for these immune reactions, reagents must be employed which affect the relevant T cell populations. One approach to this problem has been the use of anti-idiotypic antibodies against monoclonal anti-MHC antibodies to modify transplantation responses to alloantigens. These antibodies did not have a profound effect on T cell reactivity. Therefore new reagents against the TcR and other differentiation antigens on relevant T cell populations are being developed which might be useful in modifying T cell responses to alloantigens.

Proposed Course of Research: Future studies will be designed to examine the precise nature of the antigenic determinants recognized by alloreactive cloned CTL. In addition, newly derived monoclonal anti-T cell antibodies will be examined to determine if any of these antibodies might be useful in modifying transplantation responses. In addition, the newly derived monoclonal anti-T cell antibodies will be used to determine which T cells are indeed critical in responses to transplantation antigens in vivo.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05114-03 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Sequence Organization of Class I Major Histocompatibility Genes

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

PI:	D. S. Singer	Senior Investigator	IB, NCI
Others:	S. Rudikoff	Senior Investigator	LG, NCI
	R. Ehrlich	Visiting Fellow	IB, NCI
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The aim of this work is to determine the DNA sequence organization of class I genes contained in the swine major histocompatibility complex (SLA). Both genomic lambda and cosmid libraries have been constructed from a homozygous SLA^{dd} animal. From these, porcine class I SLA genes have been isolated and characterized. Three genes have been sequenced in their entirety; three others are currently being sequenced. The organization of the class I SLA genes is similar to that of other class I genes consisting of 8 exons encoding a leader polypeptide, three extracellular domains, a transmembrane and intracytoplasmic domain. Homologies between the SLA genes range from 85% between the two genes encoding the classical transplantation antigens to 60% between the most divergent genes. Although a variety of single nucleotide changes are observed in the exons of the genes, complex alterations are also seen, consistent with the interpretation that some mechanism of gene conversion may operate to generate polymorphism in this family.

Project Description

Objectives: This laboratory has undertaken a study of the molecular biology of the class I genes of the major histocompatibility locus of the miniature swine. It is known that the antigens encoded by these genes are highly polymorphic and responsible for both graft rejection and regulation of immune responses. Biochemical studies have demonstrated that the MHC antigens of mouse, man, and miniature swine are structurally homologous. As a result of studies of the MHC genes of mouse and man, others have proposed various mechanisms for the generation of polymorphism in this multigene family. The aim of this study is to attempt to assess the various selective pressures exerted on MHC genes during their evolution, including those operating at the allelic and species levels. In addition, we hope to gain further insights into the generation of polymorphism in this multigene family, since the number of class I MHC genes in the miniature swine is far more limited than in either man or mouse.

Methods Employed: Recombinant genomic DNA libraries were constructed in the lambda phage vector, Charon 4A, and the cosmid vector, pHC 79, using purified 20-40 kb pig DNA fragments, generated from partial restriction enzyme digests of pig liver DNA. Genomic clones containing class I MHC genes were identified by hybridization of radiolabelled probes, either human or swine, with the libraries. Once genomic clones are characterized as containing MHC genes, a detailed analysis of the DNA sequence organization of the clone is conducted. Segments of the isolated DNA are subcloned into plasmid vectors; subclones containing coding DNA sequences are then subjected to restriction enzyme mapping and direct DNA sequence analysis. Two methods of DNA sequence analysis are employed: the chemical degradation method described by Maxam and Gilbert and M13 subcloning coupled with dideoxy, enzymatic sequencing. Data are analyzed by a variety of available computer programs. The limits of the transcriptional unit have been mapped by appropriate exonuclease VII-protection experiments.

Major Findings: Three of the characterized class I SLA genes are expressed in mouse L cells, whereas the fourth is not. The two transplantation antigen genes are highly homologous in both the exon (88%) and intron (80%) segments whereas the level of homology with the others is much less, dropping as low as 40%. The gene organization consists of 8 exons encoding a leader domain, three extracellular domains, a transmembrane domain, cytoplasmic domain and 3' untranslated region. This organization is similar to those reported in other species. Putative regulatory sequences can be identified in the 5' flanking regions of all genes. A 'CAT' box (CCAAT) is found 5' of the initiation codon of all genes. Approximately 25 bp. down-stream from the 'CAT' box, one gene contains a canonical 'TATA' box (TATAA). In the same position, another gene contains the sequence TCTAA, which is also found in the human HLA A3 gene. However, one of the genes contains no TATA box at all.

Of the protein encoding exons, only the exon encoding the leader, exon 1, shows size variation. Exon 2, which encodes the first extracellular domain of the native molecule is the least homologous of the 3 exons encoding extracellular domains. Alterations in exon 2 are clustered, consistent with the possibility that they have arisen by gene conversion mechanisms. Further, complex alterations in exon 4, spanning 6 nucleotides, also argue for the presence of gene conversion mechanisms.

Comparison of the swine DNA sequences with class I genes from other species reveals a generally high conservation of exons 2, 3, 4, and 6 with lower homology in the remaining protein encoding domains. Introns are significantly less well conserved, although moderate homology is found between swine and human class I MHC genes in both introls and 3' flanking regions. Taken together, with comparisons of the deduced protein sequences, these data indicate an order of swine>human>rabbit>mouse in the relationship of class I genes.

Significance to Biomedical Research and the Program of the Institute: Genetic and cellular studies in a number of mammals, including man, mouse, and pig have demonstrated that class I MHC antigens play an integral role in the cellular interactions leading to both humoral and cellular immunity. In particular, the ability to respond to viral infection and to accept tissue grafts appears to be determined by the class I MHC antigens. Despite the clear importance of the major histocompatibility locus in the immune response, relatively little is known at the molecular level about the content or genetic organization of this multigene family. An understanding of the molecular variations of MHC genes and their corresponding antigens may afford the possibility of treating various immunodeficiency diseases by appropriate genetic manipulations.

Proposed Course of Project: Characterization of the structure of the swine MHC genes will proceed along the following lines. From the cosmid and phage libraries, it has been possible to identify the remaining members of the class I SLA family. The remaining members of the family will be sequenced. These studies should help to elucidate patterns of evolution of this family. In addition, we plan to focus on potential regulatory loci at the 5' ends of the various class I genes. Such studies should reveal common sequences necessary for the regulation of expression of multigene family, as well as identify unique regions required for the differential expression of these genes.

Publications:

Satz, M. S., Wang, L-C., Singer, D. S., Rudikoff, S.: 1985. Structure and expression of two porcine genomic clones encoding class I MHC antigens. J. Immunol. 135:2167.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05115-03 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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)*

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☐ (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, namely the class I MHC genes. The miniature swine has been chosen as an experimental system because there are only 7 members of the family. Of these 6 have been isolated. To address the question of the molecular regulation of the expression of the class I MHC genes, two approaches have been taken: 1) analysis of in vivo patterns of expression in a variety of tissues and in transfected mouse L cells and 2) characterization of regulatory elements associated with these genes. Three categories of MHC genes have been identified this way: 1) A set of closely related genes which are expressed in L cells and in nearly all somatic tissues, although at different levels. These genes encode products which are expressed on the cell surface and are able to bind a monoclonal antibody which recognizes a common determinant, also found on classical transplantation antigens. 2) A distantly related gene which is expressed both in L cells and in vivo but whose pattern of expression is distinct from that of transplantation antigens. 3) A set of genes which is not expressed in L cells.

Regulatory sequences within one of the transplantation antigen genes have been identified by generating a series of 5' end deletion mutants. The transcriptional promoter has been identified and the interferon-responsive element mapped to this region. No classical enhancer element has been identified in any segment of the gene.

Project Description

Objectives: This laboratory has undertaken to study the molecular biology of class I genes in the major histocompatibility locus in the miniature swine. These genes constitute a multigene family of only 7 members in the genome of the pig. It is known from serological studies in the mouse that class I antigens fall into two categories: transplantation antigens, which are constitutively expressed on nearly all somatic cell types, albeit to different extents, and differentiation antigens, such as Qa, T1, expressed primarily on one tissue type. Thus, although the members of this family are closely related with respect to DNA sequence, and clearly evolved from a common primordial ancestor, they are subject to different regulatory constraints. Therefore, this multigene family provides an excellent system in which to study the regulation of expression of its members. Two concurrent approaches to this problem are being used: 1) The expression of individual pig MHC genes is being studied in vivo and in mouse L cells transfected with various genomic MHC clones. An analysis of the regulatory constraints operating in the L cell system will provide insights into the regulation of expression of various members of the multigene family. Comparisons of MHC genes which are or are not expressed in these cells will further identify important regulatory regions. The patterns of expression of MHC genes in pig tissues will also be assessed and will allow us to classify genes as either transplantation antigen encoding or differentiation antigen encoding. It will then be possible to examine both the DNA sequences and regulatory factors responsible for differential expression. 2) The identification and characterization of regulatory elements in various class I MHC genes is being pursued in order to gain insights into mechanisms of regulation.

Methods Employed: Individual MHC clones have been isolated from either phage or cosmid genomic libraries by established techniques. Segments of pig DNA containing MHC-homologous sequences are isolated and purified; mouse Ltk^- cells are cotransformed with these DNA preparations and HSV tk genes. Selected transfectants containing pig DNA sequences are then assessed for expression of SLA antigens on the mouse cell surface and for SLA RNA expression. Expression of SLA antigens is monitored by indirect immunofluorescence, using a monoclonal anti-SLA antibody. RNA expression is monitored by hybridization of purified total RNA from transfectant cell lines with appropriate probes. To test for the presence of regulatory sequences, swine MHC genes have been truncated at their 5' or 3' ends and reassayed in mouse L cells for SLA expression. More refined mapping of regulatory sequences is accomplished by generating deletion mutants using Bal 31 exonuclease. Mutants thus generated are then ligated to test expression vectors to monitor regulatory and promotor activity by transfection into mouse L cells.

In vivo expression of class I genes is assessed by analyzing total RNA prepared from a variety of tissues. Analysis is either by direct Northern blot hybridization or by S1 nuclease.

Major Findings: Studies on the regulation of expression of class I MHC genes have focused primarily on one genomic clone, PD1. It has been demonstrated that mouse L cells transfected with PD1 express SLA antigen on their cell surfaces. This expression results from the selective transcription of SLA coding sequences; contiguous pig DNA sequences are not transcribed. To further characterize the DNA sequences which regulate this expression, a variety of deletion mutants have

been analyzed. Truncation of 5' flanking sequences of the SLA gene results in the loss of transcription of SLA sequences. Bal 31 mapping of the 5' flanking sequences has located the SLA promoter region, as follows. A series of Bal 31 deletion mutants were generated spanning a region of 1 kb. at the 5' end of the SLA gene. Each of these mutants was attached to the bacterial chloramphonicol acetly transferase (CAT) gene and its ability to promote CAT transcription assessed.

Treatment of SLA-transfectants with mouse interferon results in a marked increase in the levels of surface SLA^d and in the rate of gene transcription. Using the same set of Bal 31 deletion mutants described above, it has been possible to map the ultimate target of the interferon effect to the 5' flanking region of the SLA gene.

Analysis of RNA from a variety of tissues indicates that expression of at least three of the SLA genes is regulated and apparently in similar fashion. In all three cases, different levels of RNA are observed in different tissues, ranging from very little expression in testis and thymus to high levels of expression in lymphoid tissues. Thus, SLA gene expression appears to be regulated. The molecular basis of this expression is currently under further investigation. Analysis of 5', central and 3' segments of PD1 for the presence of classical enhancer sequences, as assessed by the ability to augment an SV40 promoter, failed to reveal any PD1 enhancers in either mouse L cells or lymphoid cells.

Significance to Biomedical Research and the Program of the Institute: Studies in a number of species, including the miniature swine, have demonstrated the importance of products of MHC genes in normal immune responses. Products of class I MHC genes control cellular responses leading to graft rejection and cellular immunity to virus. Products of other class I MHC genes are differentiation antigens. The expression of these different antigens can be modulated by a variety of exogenous factors, such as interferon or viral proteins, both of which may alter the immune response. Despite the importance of the MHC in the immune response, nothing is known at the molecular level about the regulation of expression of this multigene family. An understanding of the molecular basis of MHC gene expression may afford the possibility of developing therapeutic approaches which could appropriately modulate MHC gene expression to counteract the deleterious effect of infectious agents.

Proposed Course of Project: Studies on the regulation of MHC gene expression will proceed along the following lines:

(1) The regulation of class I MHC gene expression in mouse L cells will be further studied. Processive Bal 31 mutants from 5' of the PD1 promoter downstream will be generated and used to identify regulatory elements. Further characterization of the promoter region will be approached by promoter shuffling experiments between genes with different patterns of expression.

(2) Studies on the differential patterns of class I MHC gene expression in tissues will also be pursued. A variety of lymphoid and non-lymphoid pig cell lines are available, as well as pig interferon. It will therefore be possible to assess the expression of the SLA genes in homologous cells. This should allow the identification of regulatory sequences. Once such DNA segments have

been identified, it should be possible to isolate associated factors and characterize their tissue specificity and interactions with DNA.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05116-03 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Graft-Versus-Host Disease Prophylaxis in Allogenic Bone Marrow Transplantation

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

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Efforts are being directed towards the prevention or control of graft-versus-host disease in human allogeneic bone marrow transplantation. Since such graft-versus-host disease is mediated by alloreactive T cells in the inoculated marrow, reagents and techniques have been developed to remove these T cells from the marrow inoculum and to measure the extent of that depletion. To this end, several murine monoclonal antibodies specific for antigens expressed on human T cells have been developed, three of which are cytotoxic. These antibodies have been untilized, in conjunction with other depletion techniques, for complement-mediated lysis of T cells in marrow. By a clonogenic assay now available, residual T cells in marrow following such a depletion are at a level of less than 0.01% of the total cell population. Therefore a bank of such T cell depleted and characterized marrows has been generated for use in the therapy of human malignancy. With respect to the control of alloreactive T cells mediating graft-versus-host disease, studies on the origin or generation of such alloreactivity have been undertaken in murine radiation bone marrow chimeras. It has been shown that the generation is influenced by a unique interaction of T cell genotype and the T cell maturation environment. The fine specificity of human CTL has been demonstrated to be sufficient to distinguish among alpha 1 and alpha 2 domain changes of class I major histocompatibility complex molecules. Such mature alloreactive human cytotoxic T cells have been further studied with respect to cell surface molecules utilized in their interactions with target cells as the basis for therapeutic interventions with the intent of preventing tissue damage mediated by such alloreactive cytotoxic T cells. This in vitro generated information has been applied to bone marrow transplantation models in monkey and swine. Utilizing T cell depleted marrow, extended solid organ allograft survival (without exogoneous immunosuppression), but not long term tolerance induction, can be achieved in rhesus monkeys.

Project Description

Objectives: 1). To produce monoclonal antibodies directed against human T cell specific antigens; 2). To develop techniques utilizing these antibodies to deplete human T cells from marrow; 3). To apply these approaches for the prevention of graft-versus-host disease in human allogeneic bone marrow transplantation across major HLA barriers; 4). To establish a bank of characterized, T cell depleted marrow for use in human bone marrow transplantation; 5). To study the origin of the basis for graft-versus-host disease, the generation of the T cell allorepertoire; 6). To determine methods for the control of T cell mediated alloresponses in vivo (graft versus host and host versus graft reactions) by the use of monoclonal antibodies directed at cell surface molecules; 7). To explore new applications of bone marrow transplantation as therapeutic modalities (tolerance induction).

Methods Employed: Mice are immunized with human thymocytes and peripheral (mature) T cells. Hybridoma cell lines are obtained by fusion of immune mouse splenic lymphocytes with mouse myeloma cells. The cells are fused by exposure to polyethelene glycol and then cultured in selective HAT medium for two weeks. Hybridoma cells secreting anti-T cell antibodies are detected by assays for binding to plates coated with human T cells (cell-bound ELISA) and by complement-mediated cytotoxicity. Positive hybridoma cell lines are cloned. The monoclonal antibodies so produced are further characterized by differential binding and cytotoxicity on purified lymphocyte subpopulations. Such analysis includes two color immunoflourescence analysis by the Fluorescence Activated Cell Sorter utilizing biotinylated antibodies with avidin conjugates and directly fluoresceinated antibodies. Large amounts of monoclonal antibodies are raised in culture supernatants or by passage in vivo in mice to produce ascites hybridoma antibodies.

To further define the specificity of the monoclonal antibodies, the cell surface molecules for which they are specific are isolated and characterized. Cell surface proteins are labeled with ^{125}I. Labeled cells are solubilized by detergent lysis and large particle are removed by ultracentrifugation. Radiolabeled determinants are reacted with monoclonal antibodies and precipitated by Staphloccocal aureus. Molecular weight is determined by polyacrylamide gel electrophoresis.

Hybridoma antibodies specific for T cell antigens are purified following determination of the antibody subclass by radial immunodiffusion (Ochterlony reaction) with nylon wool, precipitation by ammonium sulfate (dialysis) passage over DEAE, gel separation and sizing. These characterized, purified antibodies are then utilized in T cell depletions. Initial titering for depletion by complement-mediated lysis is carried out on ^{51}Cr-labeled T cell targets with varying concentrations of antibody and complement. Antibody and complement at optimal titer are then utilized alone or in combination with other depletion techniques to determine relative efficacy of various depletion regimens. The effect of the T cell content in the starting, untreated cell population on the final residual T cell number after depletion treatment is assessed by applying the depletion procedure to cell populations with varying T cell content (peripheral blood lymphocytes, aspirated marrow and surgically resected marrow). Marrows with low numbers of contaminating T cells are

harvested by surical resection, prepared as cell suspensions in RPMI medium, T cell depleted by the optimal combination of depletion procedures utilizing antibody + complement and cryopreserved for human transplantation. Completeness of depletion is assessed by clonogenic assay and FACS analysis.

To study T cell allorepertoire generation, murine bone marrow chimeras are prepared by lethal irradiation of recipient mice and reconstituted with T cell depleted marrow from allogeneic donors. Cytolytic T cell activity is assayed in vitro by four hour ^{51}Cr release and IL 2 production is determined by a cell proliferation assay.

To determine the cellular mechanisms for, and to construct new therapeutic interventions of, in vivo T cell mediated tissue injury such as that of graft versus host disease and host versus graft responses, human $T8^+$ cytolytic T cell clones specific for H-2B xenogeneic murine antigens were generated, characterized, and injected into mice for in vivo study. Results demonstrated a $T8^+$ cellular cytotoxic basis for tissue injury mediated by human T cells. To better understand the cellular interactions necessary for this lysis-mediated tissue injury and to provide a basis for therapeutic intervention by administration of monoclonal antibodies specific for cell-cell interaction molecules, three pertubations of the usual human cell -- human target cell interaction were utilized to elucidate absolute requirements for the participation of the T8, T3, T cell receptor, T11 and LFA1 molecules on the T cell surface in the lytic event. Using the human allospecific cytolytic T cell line as a standard, these pertubations of the human T cell -- human target cell interactions have been (1) alteration of the species of the target cell, but keeping intact the role played by the antigen specific receptor (TcR.), (2) bypassing the antigen specific receptor, but maintaining a role for the T3 molecule (T3-redirected killing) and, (3) bypassing the TcR-T3 complex by PMA + calcium ionophore activation.

To study the utilization of T cell depleted (TcD) allogeneic bone marrow transplantation for tolerance induction in a setting of solid organ transplantation, we first investigated the effects of TcD autologous bone marrow transplantation. Methods of TcD of rhesus bone marrow were determined as well as the development of assays to definitively quantitate the extent of that depletion and final T cell content in the depleted marrow. Rhesus monkeys were irradiated, transplanted with their own T cell-depleted marrow, and then received an allogeneic cardiac (heterotopic) transplant. Animals were monitored for graft rejection and immunoreconstitution. Similarly, in order to accomplish such studies in a genetically defined system, such bone marrow transplantation techniques have also been applied in miniature swine.

To further study regulation of T cell alloresponses and xenogeneic responses, human T cell clones with specificity for alloantigens or xenoantigens are generated by stimulation with allogeneic or xenogeneic cells and IL2 under conditions of limiting dilution and then expanded to the numbers needed. Specificity is assessed by cytotoxic cell activity against transfected L cells, in vivo generated mutants, or in vitro generated mutants. Cell surface molecule interactions are investigated by the use of monoclonal antibodies with specificity for LFA-1, LFA-2, T3 or T8.

Major Findings: Fusion of murine myeloma cells with mouse spleen cells immunized against human T cells has resulted in eight clones, six with specificity for human T cells. Of these, three are stable hybridomas producing cytotoxic antibodies. Two of these antibodies are IgG2 and one is IgM. One antibody is blocked in its binding by the monoclonal 9.6 and inhibits E-rosette formation; by these criteria it is therefore specific for the T cell E-rosette receptor. It is pan-thymocyte in distribution, inhibits killing of allogeneic targets by cytotoxic T cells, inhibits T cell proliferation induced by allogeneic determinants and is negative on rhesus monkey lymphocytes. Another is pan-T in its distribution. It reacts with a subset of thymocytes, does not inhibit cytotoxic T cells, and is positive on Rhesus monkey lymphocytes. Preliminary evidence from immunoprecipitation studies indicates that this antibody is specific for the T cell antigen receptor.

Optimal T cell depletion of heavily T cell contaminated populations results when antibody plus complement lysis is preceeded by E-rosetting. Two cycles of complement are distinctly superior to one. Surgically resected marrow with one to five percent contamination by cells bearing mature T cell markers has fewer residual T cells after a fixed depletion regimen of E-rosetting followed by antibody plus complement than aspirated (conventionally harvested) marrow with ten to thirty percent contamination by mature T cells. Resected marrow after T cell depletion has a residual T cell content of less than 0.01% by the clonogenic assay. This assay detects as few as 1 to 2 T cells per $1x10^6$ marrow cells. Marrow can be surgically resected from organ donors with yields of greater than 10^{10} cells which is adequate for consideration of clinical application. Such marrows are now available for use in a therapeutic protocol which is open for patient treatment.

The H-2^b T cell repertoire specific for the allodeterminants encoded by the K^b mutant bm6 results from a unique interaction of T cell genotype and T cell maturation environment. This interaction involves gene products encoded by the K region with the result that K region homology is required for the generation of the repertoire. This raises the possibility of class I intramolecular associative recognition requirements in T cell responses to alloantigens. Recent experiments further demonstrate that class II homology is also necessary and that an important role is played in these chimeric allospecific responses against bm6 by $T4^+$ class II specific helper cells. The lack of $Lyt2^+$ helper T cells similarly plays a key role in the lack of responsiveness to the bm6 alloantigen in certain chimeric combinations.

Xenoreactive T cells specific for class I determinants can be cloned directly from an unsensitized peripheral human lymphocyte population expanded to numbers sufficient to study mechanisms of xenoreactivity, recognition of xenogeneic target cells, and to evaluate comparability to allogeneic interactions. These human T cells have sufficient specificity to distinguish alpha 1 vs. alpha 2 domain associated antigens of class I major histocompatibility complex molecules. Studies on the role of the T cell surface interaction molecules indicates that LFA2, T3 and epitopes of T8 play variable rather than fixed (absolute) roles, that LFA1 and LFA2 may provide "off" signals, that the latter may convey activation signals as well via the same epitopes, that T cells appear to rely entirely on either a CD2 or CD18 pathway in cell-cell contact, and that the CD8 ligand is unlikely to be the class I molecule of the

target cell. Based on current results, a combination regimen of anti-LFA2 and anti-LFA1 monoclonal antibodies would provide a potent therapeutic intervention for T cell mediated events in vivo.

T cell depletion of rhesus marrow showed low numbers of residual T cells in the injected marrow (less than or equal to 0.008%), that residual T cell content correlated with early graft rejection, that recovery of the $T4^+$ subset in the reconstituted animals is prolonged in both TcD and non TcD transplanted monkeys, that $T8^+$ cells recover early during reconstitution, that graft rejection correlates best with recovery of $T4^+$ cells in the setting of a functional $T8^+$ subset, and that graft survival is significantly prolonged (without further immunosuppression) in the animals receiving T cell depleted marrow.

Significance to Biomedical Research and the Program of the Institute: Monoclonal antibodies directed against human T cell specific antigens can be used to probe the biological functions of these antigens and to eliminate T cells bearing these antigens from marrow for the purpose of preventing graft-versus-host disease in human allogeneic bone marrow transplantation.

The prevention of graft-versus-host disease and the establishment of a bank of characterized T cell depleted marrow for use in clinical bone marrow transplantation will provide a significant, new potential for the use of allogeneic bone marrow transplantations as a treatment modality for a variety of clinical diseases including neoplasms, inborn errors of metabolism and hemoglobinopathies. An understanding of the generation of the T cell allo repertoire is intertwined with issues of T cell recognition of alloantigens. Control of such alloresponses which are the basis of graft-versus-host disease will depend on an understanding of T cell function and recognition requirements. The mechanisms of target cell/alloreactive T cell interactions provides a model of the formulation of new therapeutic approaches aimed at preventing the tissue damage associated with the interaction of human CTL with their targets.

Proposed Course of Project: Efforts will continue to generate monoclonal antibodies which are T cell specific in their activity. The biological relevance of the T cell antigens towards which such antibodies are directed will be assessed in a variety of systems such as T cell proliferative responses and T cell mediated cytotoxicity.

Techniques for depletion of T cells from marrow will be further expanded and refined. The use of a variety of plant and bacterial toxins is being investigated.

A bank of T cell depleted marrows has been established with characterization as to HLA typing, extent of T cell depletion and viability of marrow progenitor cells. Clinical utilization of these marrows in the treatment of malignant disease amenable to bone marrow transplantation therapy for patients without HLA matched donors is underway.

Efforts will continue in the use of murine radiation bone marrow chimeras for studies on the generation of the T cell allorepertoire and T cell recognition of alloantigens.

Alloreactive and xenoreactive T cell clones and lines will be utilized for the study of the cellular interactions necessary for tissue injury in vivo. Monoclonal antibodies specific for T cell surface antigens in combination with perturbations of the usual human effector cell-target cell interaction will be used to better understand the factors crucial to the T cell mediation of graft vs. host disease and graft rejection in vivo in man.

Publications:

Sharpe, T. A., R. E. Gress, D. H. Sachs, and S. A. Rosenberg: 1985. Assessment of mixed lymphocyte reactivity and human bone marrow cell cultures. Transplantation 40:551.

Moses, R. D., R. E. Clark, P. J. Lucas, R. R. Quinones, K. S. Yaekel, D. H. Sachs, and R. E. Gress: 1986. Immunological reconstitution and cardiac allograft survival in rhesus monkeys conditioned with irradiation and T cell-depleted autologous marrow transplantation. In Recent Advances in Bone Marrow Transplantation, R. P. Gale and R. Champlin, editors. Alan R. Liss, Inc., New York.

Sakamoto, K., L. R. Pennington, F. A. Popitz-Bergez, M. D. Pescovitz, R. E. Gress, M. A. McDonough, S. Shimada, K. I. Katz, and D. H. Sachs: 1986. Swine GVHD model and the effects of T cell depletion of marrow by monoclonal antibodies. In Recent Advances in Bone Marrow Transplantation, R. P. Gale and R. Champlin, editors. Alan R. Liss, Inc., New York.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05117-03 I

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October 1, 1985 to September 30, 1986

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

Allodeterminants of Class I Major Histocompatibility Antigens

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

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Current efforts have been devoted to examining the nature of the allo-determinants recognized by cloned T cell populations. To examine this question, H-2 structural mutants have been isolated from a somatic cell line by mutagenesis and immunoselection using monoclonal anti-H-2 antibodies. Examination of alloantigen-specific CTL clones on these mutants suggest that the majority of CTL clones recognize determinants different from those which elicit antibody production. Analysis of the in vitro-derived mutants has shown that new determinants are created by the mAb immunoselection procedure which can be recognized by cytotoxic T cells. The new allodeterminants expressed on the in vitro-derived CTL can function as transplantation antigens in vivo and appear linked to a single amino acid substitution. In addition, the regions of the MHC molecule involved in CTL recognition were studied using L cells transfected with H-2 genes constructed by shuffling exons between the H-$2L^d$ and H-$2D^d$ genes. The findings suggested that unlike mAbs which can recognize individual epitopes on different domains, a majority of the CTL clones recognize determinants influenced by the interaction of the two external domains. In one instance, CTL can recognize a hybrid D^d/L^d molecule (T9-10-3). However, the CTL employ a restricted TcR V_β chain family. Other altered MHC class I genes have also been examined including L cells which have been transfected with truncated L^d and D^d genes and express only the $\alpha 3$/TM portion of the molecule. The finding demonstrated that CTL can be generated against truncated MHC gene products. Finally, L cells transfected with hybrid MHC genes between mouse and human class I genes have also been examined. In some instances, CTL clones which recognize native K^b-transfected L cells do not recognize MHC hybrid molecules that have human α_3 domain. Thus, the specificity of CTL although predominently determined by the $\alpha_1 + \alpha_2$ domains is critically influenced by α_3.

Project Description

Objectives: Monoclonal alloreactive T cell populations have been generated to: (1) Identify the regions of class I MHC heavy chains that are responsible for the alloantigenicity of these molecules; (2) investigate the activation requirements of T cells; and (3) determine the role of conformation of the H-2 molecule in induction and blocking of alloresponses.

Methods Employed: Cloned CTL were isolated from alloreactive mixed lymphocyte cultures. CTL were cloned by limiting dilution at 1 cell/well in the presence of stimulator cells and 10% T cell growth factor (IL-2) and screened for cytolytic activity in a 4 hour ^{51}Cr release assay. Fine specificity of the cloned CTL was examined using a panel of MHC congenic mouse strains and in some cases MHC mutant mice.

Somatic cell lines, from which mutants were selected, were derived from F_1 neonatal bone marrow cells transformed with Abelson virus. Monoclonal antibodies reacting with different epitope clusters were used to immunoselect H-2 expressing tumor cells. Mutagenized cells were treated repeatedly with a mAb plus complement. The minority of residual cells which expressed normal amounts of cell surface MHC were positively selected by fluorescence activated cell sorting using a pool of mAbs and subcloned using soft agar. Clones with reduced reactivity with the selecting antibody but near normal reactivity with other anti-H-2 mAbs were used.

One type of hybrid class I genes were constructed by reciprocal exon exchanges between the L^d and D^d genes. The resultant constructs which had their α_1 domains exchanged were transfected into L cells. These transformed cell lines expressed a polypeptide with domain α_1 of L^d and all other domains from D^d (L^d1/D^b) or reciprocally domain α_1 of D^d and all other domains from L^d (D^d1/L^d). Additional truncated H-2 class I genes were constructed by deletion of the $\alpha1$ and $\alpha2$ domains of the D^d and L^d molecule. These transformed cell lines expressed a smaller polypeptide with the $\alpha3$ domains apparently unaltered and expressed on the L cell surface. Transfected recombinant HLA-A2 and H-$2K^b$ genes were also transfected in mouse L cells and a human rhabdosarcoma cell.

Cytolytic T cell reactivity was examined in a ^{51}Cr-release assay. Target cells were radiolabeled with ^{51} Chromate solution. Effector cells were added and the mixture incubated for 4 hrs. The supernatants were collected and percentage lysis was calculated. Skin graft analysis was performed by transplanting full thickness tail skin to recipient mice. The grafts were assessed for rejection on a daily basis.

Major Findings: In vitro-derived H-$2K^b$ structural variant lines were studied using cytotoxic T cell (CTL) clones generated from different alloreactive combinations and monoclonal antibodies (mAbs) which reacted with different H-$2K^b$ domains. The H-$2K^b$ structural mutants were derived from a mutagenized somatic cell line (R8) using an α_1 or an α_2 domain specific mAb as the immunoselecting reagents. The determinants recognized by the CTL clones generated in different allogeneic combinations were altered in some of these mutant cell lines. It is clear from the analysis that the determinants

recognized by the CTL and mAbs are different. However, there appears to be some conformational changes which affect both specificities. mAb blocking studies suggested that inhibition of CTL activity by anti-H-2K^b mAbs does not necessarily reflect the fine specificity of ther CTL activity. Finally, neo-determinants were identified on the in vitro-derived somatic varients. CTLs generated from a wild type C57BL/10 anti-H-2K^{bm3}, H-2K^{bm23} or H-2K^{bm6} mutant CML reaction lysed two of the R8 mutants, R8.24 and R8.246 but did not lyse the wild type R8 cells. Additional evidence suggests that one amino acid, No. 80, has been altered in the R8.246 mutant. This amino acid has not been altered in the H-2^{bm3} and H-2^{bm23} in vivo mutant mice. However, both these mutants have altered amino acid 77. Thus this region of the molecule appears to be highly relevant for CTL recognition. In vivo injection of R8.246 results in priming for skin graft rejection of a subsequent bm3 graft. Thus, the novel epitopes expressed on R8.246 appear to create transplantation antigens.

To identify the regions of class I antigens that are involved in CTL recognition, mAbs and CTL clones specific for either H-2L^d or H-2D^d have been examined on the H-2 hybrid antigens (D^d_1/L^d and L^d_1/D^d) expressed on transfected L cells. The pattern of CTL and mAb recognition suggests that the interaction of the first two domains influences the allogeneic determinants. The binding of all but one of the mAbs could be mapped to one of the external domains, α_1 or α_2, although the binding affinity in several instances was markedly reduced. By comparison, only a minority of the CTL clones and bulk CTL populations examined lysed the transfected cells, suggesting that either the affinity of the CTL recognition was reduced sufficiently to obviate lysis or the determinants recognized by the CTL were sufficiently altered to prevent CTL recognition. In one instance, CTL have been identified that lyse the hybrid MHC molecule, and the reactive CTL clones use the same Vβ chain of the TcR. Thus, a correlation may exist between β chain usage and allospecificity. Although the α_1 and α_2 domains are critical in T cell recognition, the α_3 domain is also involved. Specific K^b-specific CTL clones recognized H-2K^b/HLA-A2 recombinant MHC molecules, in which the α_1 and α_2 domains were H-2K^b and the α_3 domain was HLA-A2. However, the reactivity was significantly lower then the reactivity of the same CTL on $K^b_{\alpha 1+2}/D^b_{\alpha 3}$ or K^b transfectants. Several clones which recognized K^b in L cells did not recognize the K^b/A2 molecule in the L cell lines. Additional studies using truncated H-2D^d and H-2L^d transfected L cells showed that specific CTL could be generated against α_3 domain of the class I molecule. Thus the α_3 domain is clearly involved in CTL recognition.

The MHC regions involved in the rejection of skin grafts was examined. Mice were primed, in vivo, with L cells transfected with hybrid MHC molecules (L^d/D^d). Skin graft rejection was assessed across a class I-only L^d difference. The findings suggested that the α1 and α2 domains were most important in skin graft rejection and the immune cells which mediate the rejection recognize conformational determinants dependent on both the α1 and α2 domains.

Significance to Biomedical Research and the Program of the Institute:
Alloreactive T cell responses provide the major barrier to successful organ transplantation. In order to specifically modulate alloreactivity a greater understanding of the nature of allorecognition is required. These studies suggest that allodeterminants recognized by cytolytic T cells differ from those recognized by alloantibodies. In addition, recognition depends heavily on the

conformation of the class I molecule. Finally, single amino acid changes can result in new determinants that can function as transplantation antigens.

Proposed Course of Research: New H-2 mutants and class I hybrid gene constructs will be examined to further define the polymorphic structures on MHC molecules involved in allorecognition.

Publications:

Bluestone, J. A., Foo, M., Allen, H. and Flavell, R. A.: Allospecific cytolytic T lymphocytes recognize conformational determinants on hybrid mouse transplantation antigens. J. Exp. Med. 162:268-281, 1985.

McCluskey, J., Bluestone, J. A., Coligan, J., Macoy, L. and Margulies, D. H.: Serologic and T cell recognition of transfected transplantation antigens encoding by in vitro deleted class I major histocompatibility genes. J. Immunol. 136:1472-1481, 1985.

Bluestone, J. A., Langlet, C., Geier, S., Nathenson, S. G., Foo, M. and Schmitt-Verhulst, A.-M.: Somatic cell mutants expressing structural variants of the H-2K^{b} molecule express altered allodeterminents recognized by cytolytic T-cell clones. J. Immunol., in press (1986).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05118-03 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line betwaen the borders.)*
Regulation of Immune Response to Tumor Cells and Alloantigen

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

PI:	C. C. Ting	Senior Investigator	IB, NCI
Others:	M. E. Hargrove	Microbiologist	IB, NCI
	N.-N. Loh	Summer Student Volunteer	IB, NCI
	S. S. Yang	Senior Investigator	LCO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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

B

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

I. Regulation and Control of Immune Responses:

A. Production and characterization of T cell differentiation factor (TCDF). TCDF was produced in syngeneic lymphocyte-macrophage culture. The production of TCDF required the recognition of self Ia on macrophages, and the presence of L3T4 cells. In addition to interleukin 2 (IL2), TCDF was also needed to induce the differentiation of mitogen-activated CTL (cytotoxic T lymphocytes) into CTL effectors. Upon separation by Sephadex G100 gel filtration, the peak TCDF activity was at 16,000 dalton mw, which was distinct from IL2 whose peak activity was at 30,000 dalton.

B. Regulation of the cytotoxic activity of alloreactive CTL by helper cells and lymphokines. Transferring donor cells from allo-CTL generated in unseparated mixed lymphocyte culture (MLC) into host culture (HC) containing syngeneic lymphocytes and macrophages resulted in the generation of allo-CTL of donor origin. In contrast, transferring cultured cells generated in Lyt2-depleted MLC into HC resulted in the generation of allo-CTL of host origin. Two different helps were identified here. Antigen nonspecific help was generated in the host culture, and antigen specific help was generated in Lyt2-depleted MLC. Both required L3T4 cells.

II. Tumor Immunology: LICC (lymphokine-induced cytotoxic cells) or LAK (lymphokine-activated killer) cells were generated by culturing normal spleen cells with syngeneic macrophages and indomethacin, or by culturing normal spleen cells with exogeneous IL2. The precursors of LICC were $Thy1^-$, Asialo $GM1^+$, $Lyt2^-$ and thus were NK-like cells. The effectors were $Thy1^+$, Asialo $GM1^-$, $Lyt2^-$ and thus were neither classical NK cells nor classical CTL. LICC selectively killed lymphoid and tumor targets of different etiological origin, and these cells might represent an important alternate host defense mechanism against tumor growth.

Project Description

Objectives: 1) Regulation of immune responses to tumor cells; 2) Regulation of immune responses to alloantigen; 3) Regulation of lymphokine production for the generation of cytotoxic lymphocytes; 4) Expression and function of Asialo GM1 on CTL and LAK cells.

Methods Employed: 1) Tumor cells used in experiments: Tumor cells transformed by oncogenic viruses, chemicals or unknown agents are kept in tissue culture or maintained as transplanted tumors in mice; 2) In vitro cell-mediated immunity was determined by the ^{125}IUdR release assay; 3) In vivo tumor immunity was determined by the tumor neutralization experiments; 4) In vitro generation of cytotoxic effectors. The allogeneic mixed lymphocyte cultures were used to generate the alloreactive cytotoxic T lymphocytes (CTL), the lymphokine-induced cytotoxic cells (LICC) were generated either by culturing normal spleen cells with syngeneic peritoneal cells and indomethacin, or by culturing normal spleen cells with interleukin 2 (IL2) and cytotoxic cell differentiation factors (CCDF); 5) Lymphokine production: Purified Interleukin 1 (IL1) was purchased from Genzyme (Boston, MA). CCDF was prepared by culturing mouse peritoneal cells with indomethacin. TCDF (T cell differentiation factor) was prepared by culturing normal mouse spleen cells with peritoneal macrophages and indomethacin. Purified human recombinant IL2 was obtained from Dr. Steven A. Rosenberg (Surgery Branch, NCI); 6) Monoclonal antibodies: α Thy1.2, α Thy 1.1, α Lyt2.2, α L3T4a were culture supernatants from various hybridoma lines; 7) α Asialo GM1 antibody was purchased from Wako Chemical, Dallas, TX.

Major Findings:

1. Regulation and Control of Immune Responses:

A. Lymphokine regulation of the generation of cytotoxic T lymphocyte (CTL): In addition to the signal provided by mitogen leukoagglutinin (LA), at least two additional signals were needed to generate CTL. They were interleukin 2 (IL2) and T cell differentiation factor (TCDF). After activating the splenic CTL precursors (CTLp) by LA, either purified IL2 or TCDF alone was unable to induce the differentiation of CTLp into CTL effectors. Both lymphokines (IL2 and TCDF) were required to complete this process.

B. Production of TCDF: TCDF was produced in syngeneic lymphocyte-macrophage cultures containing normal spleen cells, peritoneal adherent cells and indomethacin. a) Indomathacin was needed to block the endogenous production of prostaglandin which could suppress the production and function of various lymphokines, including IL2 and TCDF. b) Recognition of self Ia molecules was essential for initiating the TCDF production. Adding monoclonal antibody against IA or IE locus blocked the TCDF production. c) Depletion of macrophages from the cultures totally abolished TCDF production. The role of macrophages could not be replaced by IL1. d) In addition to macrophages, L3T4 cells were also needed to produce TCDF. e) Production of TCDF did not require exogenous protein. TCDF could be produced in serum free medium.

C. Partial purification of TCDF: Conditioned medium containing TCDF and IL2 was precipitated by 70% ammonium sulfate. the precipitate was dialysed

and passage through Sephadex G100 gel filtration. IL2 was eluted first and the peak activity was at 30,000 dalton m.w. The peak TCDF activity was found to be at 16,000 dalton m.w.

D. Regulation of the cytotoxic activity of alloreactive CTL by helper cells and lymphokines: The cytotoxic activity of allo-CTL was maintained and augmented by transferring cells from a 5-day mixed lymphocyte culture (MLC) into host culture (HC) containing indomethacin, freshly explanted normal spleen cells and syngeneic peritoneal cells. Transferring unseparated MLC cells resulted in the generation of allo-CTL of donor (MLC) origin. Transferring Lyt2-depleted MLC cells resulted in the generation of allo-CTL of host (HC) origin. These experiments demonstrated that antigen nonspecific help was generated in the host culture and an antigen specific help was generated in Lyt2-depleted MLC. L3T4 cells were required in both cases.

E. Expression and function of asialo GM1 on CTL and lymphokine-activated killer (LAK): There was a differential expression of asialo GM1 on CTL and LAK cells. Asialo GM1 was expressed on the precursors of LAK but was undetectable in 5-6 day cultured LAK effectors. In contrast, asialo GM1 was not detected on the CTL precursors but was readily detectable in 3-7 day cultured CTL effectors and some cloned CTL.

2. Tumor Immunology:

Characterization of the precursors, effectors and regulatory ancillary cells involved in lymphokine-induced cytotoxicity: Lymphokine-induced cytotoxic cells (LICC) or lymphokine-activated killers (LAK) were induced by culturing normal spleen cells with syngeneic peritoneal macrophages and indomethacin, or by culturing normal spleen cells with exogenous IL2. the precursors were found to be $Thy1^-$, $Lyt2^-$ and asialo $GM1^+$ cells. The effectors were $Thy1^+$, $Lyt2^-$ and asialo $GM1^-$ cells. To induce LICC, macrophages were needed to produce a cytotoxic cell differentiation factor (CCDF), and helper T cells were needed to produce IL2. $Lyt2^+$ T cells down-regulated LICC responses.

Significance to Biomedical Research and the Program of the Institute:

1. The lymphokine-activated killer (LAK) cells have been shown to be effective in clinical trials in immunotherapy of some cancer patients. However, more than half of the patients receiving LAK cells were resistant to the treatment. Therefore, it is important to examine the mechanisms for the generation of LAK cells. The characterization of the precursors, effectors and regulatory ancillary cells should help to determine effective ways to generate LAK cells.

2. IL2 was shown to be essential for the generation of CTL and LAK cells. However, the amounts of purified IL2 used in the in vitro experiments were well above the physiological levels. the large amount of IL2 used in clinical trials often induced toxic effect. We found that the addition of differentiation factors (CCDF or TCDF) reduced the requirement of IL2 for the generation of LAK or CTL. Therefore, isolation and purification of these factors may prove useful in clinical use of IL2.

Proposed Course of Research:

1. Regulation and control of immune response: lymphokine-regulation of the generation of CTL and LAK cells.

2. Expression and function of asialo GM1 on CTL and LAK cells.

3. Generation of LAK in tumor bearing hosts and its implication in cancer immunotherapy.

4. Purification of TCDF.

Publications:

Ting, C. C., Yang, S. S., and Hargrove, M. E. Lymphokine-induced cytotoxicity: characterization of effectors, precursors, and regulatory ancillary cells. Cancer Res. 46:573-578, 1986.

Ting, C. C., Hargrove, M. E. and Loh, N.-N. Production of T cell differentiation factor in syngeneic lymphocyte-macrophage culture for cytotoxic T lymphocyte generation. J. Immunol. 136:1726-1733, 1986.

Ting, C. C., Loh, N.-N. and Hargrove, M. E. Regulation of the cytotoxic activity of alloreactive cytotoxic T lymphocytes by helper cells and lymphokines. Cellular Immunol., in press, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05119-03 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Role of Helper T Cells in Allogeneic Responses

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

PI:	T. Mizuochi	Visiting Fellow	IB, NCI
Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In the last year, we demonstrated that there were at least two distinct subpopulations of helper T cells that initiate the generation of allospecific CTL responses, including (1) $L3T4^+Lyt2^-$ Th that is class II MHC-restricted, and (2) $L3T4^-Lyt2^+$ Th that is class I MHC-restricted. The recognition repertoire of $Lyt2^+$ Th was very narrow in that $Lyt2^+$ Th cells were activated exclusively by class I alloantigens. In contrast, the recognition repertoire of $L3T4^+$ Th was broad in that $L3T4^+$ Th cells responded to class I alloantigens, class II (self or allo) antigens, as well as minor-H and TNP-modified self MHC antigens. Precursor frequency of $Lyt2^+$ Th cells was found to be similar to that of $L3T4^+$ Th cells, suggesting the biological significance of this novel Th subset. In fact, it was demonstrated that $Lyt2^+$ subset played an essential role in the rejection of allo-class I disparate skin grafts in vivo. Finally, it was indicated that functionally distinct but phenotypically identical $Lyt2^+$ Th and $Lyt2^+$ CTL differed in their differentiation pathway, even though they are both class I-specific, and this might explain the difference between their fine recognition specificities defined by mutant class I stimulators.

GPO 914-918

Project Description

Objectives: The major objective of this project is to define the recognition specificity, relative precursor frequency, and the in vivo relevance of class I-specific IL-2 secreting helper T (Th) cells. Repertoire difference between $Lyt2^+$ Th and $Lyt2^+$ CTL is also studied.

Methods Employed: CTL assay: Mixed lymphocyte cultures consisted of: a) $4x10^6$ responder spleen cells, b) $4x10^6$ irradiated (2000 rad) stimulator spleen cells, and c) $4x10^6$ T-depleted and irradiated spleen cells as a source of accessory cells. After 5 days of culture, cytotoxic activities of effector cells were assayed on the relevant target cells by standard 4 hr ^{51}Cr-release assay.

IL-2 assay: Culture supernatants were assayed for IL-2 content by their ability to support the proliferation of the IL-2 dependent T cell line (HT-2). Proliferation was assessed by the incorporation of ^3H-thymidine.

Limiting dilution analysis: 24-48 replicate cultures containing varying number of responder cells and $5x10^5$ stimulator cells were set up in microculture plate. Minimal estimates of Th and CTL frequencies were calculated by analysis of the Poisson distribution relationship between the percentage of negative cultures and the number of responder cells.

Major Findings:

1. $Lyt2^+$ Th cells are stimulated only by class I alloantigens, but not by self- or allo-class II MHC antigens, minor-H antigens, or TNP-modified self MHC antigens.

2. Precursor frequency of $Lyt2^+$ Th cells is similar to that of conventional $L3T4^+$ class II-restricted Th cells.

3. $Lyt2^+$ Th cells are found to be essential for the rejection of class I-disparate allo skin grafts in vivo.

4. $Lyt2^+$ Th and $Lyt2^+$ CTL seem to differ in their requirements for intrathymic differentiation.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the T-helper mechanisms which initiate alloreactions should provide us with the tools to selectively augment or decrease immune reactivity in various clinical states.

Proposed Course of the Project: In the future, this project will be directed at investigating the biological significance of $Lyt2^+$ Th subset, especially its role in alloreaction in vivo. The repertoire difference between $Lyt2^+$ Th and CTL is also the subject of further investigation.

Publications:

Mizuochi, T., Golding, H., Rosenberg, A. S., Glimcher, C. H., Malek, T. R., and Singer, A.: Both $L3T4^+$ and $Lyt2^+$ T helper cells initiate CTL responses against allogeneic MHC antigens but not against TNP-self. J. Exp. Med. 162:427, 1985.

Mizuochi, T., Ono, S., Malek, T. R., and Singer, A.: Characterization of two distinct primary T cell populations that secrete IL-2 upon recognition of class I or class II MHC antigens. J. Exp. Med. 163:603, 1986.

Morrissey, P. J., Sharrow, S. O., Kohno, Y., Berzofsky, J. A., and Singer, A.: Correlation of intrathymic tolerance with intrathymic chimerism in neonatally tolerized mice. Transplantation 40:68-72, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05120-03 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

The Regulation of Lymphocyte Proliferation

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

PI:	K. Kelly	Senior Investigator	IB, NCI
Others:	M. Kearns	Guest Researcher	IB, NCI
	S. Irving	Guest Researcher	IB, NCI

COOPERATING UNITS *(if any)*

Ulrich Siebenlist, Laboratory of Immune Regulation, NIAID, NIH

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	1

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The research of this laboratory is directed at understanding mechanisms of tissue and growth-specific gene regulation in T lymphocytes. Lymphocyte metabolism and effector function expression are regulated by antigen/mitogen and lymphokine binding to cell surface receptors. We are investigating the physiological consequences of mitogen and lymphokine mediated signals by isolating and characterizing genes which are transcriptionally regulated by these events. We expect that genes induced within a few hours after antigen or mitogen activation of lymphocytes will be fundamentally important for the initiation of proliferation and effector function expression in these cells. We have shown that the c-myc oncogene is transcriptionally induced as early as one hour after the activation of murine B cells with LPS or T cells with Con A. Two additional proto-oncogenes, c-myb and c-fos, are similarly regulated by both IL-2 and PHA or PHA only, respectively, in human T lymphocytes. Thus, three oncogenes are members of the gene family regulated by mitogen binding to the surface of lymphocytes. The identification of additional members of this inducible gene family has been made utilizing PHA stimulated human peripheral blood T cells and subtraction cDNA cloning methodology. An additional gene sequence, the T cell receptor β chain, that encodes a product fundamental for the manifestation of effector function in T cells currently is being analyzed with regard to DNA sequences and trans-activating factors that determine expression.

Project Description

Objectives: The broad objectives of this laboratory are directed at understanding mechanisms of gene regulation. The developmentally-regulated expression of the T cell-specific gene sequence, the T cell receptor β chain, is being studied for the purpose of determining which DNA sequences and trans-acting factors regulate specific expression. In addition, the isolation and characterization of gene sequences that are transcriptionally activated in mitogen-stimulated T cells is being pursued. Characterization of inducible gene sequences will include 1) structural analysis and positive identification with known gene sequences/gene products where possible and 2) analysis of induced genes for regulatory properties such as kinetics of induction, cell cycle expression and the mechanism of RNA level increases. Specific emphasis is placed upon analysis of DNA regulatory sequences that control growth-specific expression.

Methods Employed: We are utilizing recombinant DNA technology that is useful in the manipulation of eucaryotic RNA and DNA. Some of the specific methods employed include subtraction cDNA cloning, genomic cloning, Northern and Southern blot analyses and S1 nuclease analyses. The methodology employed for DNA-mediated gene transfer includes DEAE-Dextran transfection, retroviral infection, and standard tissue culture techniques.

Major Findings: As a new laboratory, much of our effort in the past six months has concerned the development of new techniques and systems (sections B and C) that will form the basis for future investigation.

A. The progression of T cells from GO to DNA synthesis requires at least two temporally separated growth signals; one initial signal resulting from antigen or mitogen binding, and the other later signal from interleukin 2 binding. The effects of such activation signals appear to be mediated in large part by the expression of novel gene sequences. The steady-state RNA levels of three proto-oncogenes, c-myc, c-fos, and c-myb, are elevated very soon after the delivery of growth signals to T cells. In human peripheral blood lymphocytes, c-myc, c-myb, and c-fos RNA levels are induced within 30 and 90 minutes, respectively, in response to PHA or OKT3 binding. On the other hand, c-myc and c-myb RNA transcripts increase within 60 minutes following IL-2 binding, while c-fos expression is not induced. The elevation of these gene transcripts occurs in the presence of cycloheximide, indicating a mechanism of regulation independent of protein synthesis. An important question concerns the mechanism of regulation that determines increases in steady-state levels of mRNA. Increased mRNA half-life, increased transcriptional initiation, or a combination of these two mechanisms could account for elevated levels of cytoplasmic mRNA. We have investigated the mechanism of regulation for the three nuclear oncogenes, c-fos, c-myc, and c-myb, after mitogenic stimulation of primary PBL by mitogens. As shown by nuclear run-on transcription assays, there is a dramatic increase in total transcriptional rate for c-fos and c-myc but not c-myb following PHA stimulaiton, and an increase in both c-myc and c-myb after IL-2 addition to cells. Thus, c-myc and c-fos regulation occurs in large part as a result of transcriptional control in the nucleus. The mechanism of c-myc regulation appears to be similar for the two ligands, IL-2 and PHA. In contrast, c-myb mRNA accumulation appears to result from a post-transcriptional

alteration in response to PHA and at least in part from a transcriptional mechanism is response to IL-2.

The finding that c-myc is transcriptionally regulated is a basis for further experimentation to determine the DNA sequence(s) that control such regulation in response to exogenous growth signals. The experimental approach we have taken involves the introduction of genetically altered c-myc genes into fibroblasts and lymphoid cells that regulate endogenous c-myc genes in response to mitogens. Therefore, we have constructed a variety of genetically engineered c-myc genes that contain a human c-myc cDNA encoding the body of the normal mRNA contiguous with various upstream sequences that appear to be regulatory in nature by virtue of the fact that these sequences define chromatin hypersensitivity sites. These constructs have been subcloned into retroviral vectors that allow efficient gene transfer into the cells of interest. Retroviral stocks have been generated from $CaPO_4$-transfected packaging cells by conventional methods.

B. The genetic regulatory mechanism that determines tissue-specific expression in T cells is being addressed utilizing as a representative example the T cell receptor β chain. the experimental approach that has been taken involves the introduction via DNA-mediated gene transfer of intact and genetically engineered β chain genes into fibroblasts and various hematopoietic cells in order to investigate the variety of tissues that allow expression, and those DNA sequences that regulate such expression. A vβ1 containing murine β chain genomic gene was obtained as a phage clone and subsequently subcloned into a small plasmid vector to allow restriction enzyme analysis and into an expression vector utilized for DNA-mediated transfection. An extensive restriction map has been determined and various gene constructions have been initiated that remove putative regulatory sequences. Sequencing data has been obtained that allows a preliminary assignment of promotor sequences. Also, an optimal transfection protocol for T cells has been established as has an S1 nuclease assay procedure that specifically and sensitively detects transcripts encoded by the exogenously-introduced β chain gene.

C. A fundamental question concerning lymphocyte activation is the identification of those gene sequences that are transcriptionally regulated following mitogen or antigen signalling. The isolation of such genes will enable their identification and the characterization of the mechanisms by which they are regulated. We are currently isolating cDNA clones that are induced in T lymphocytes within four hours after PHA stimulation.

The ability to isolate an inducible gene depends upon the differential expression of that gene in induced and noninduced messenger RNA populations. Although it is expected that the gene induced as a result of mitogen stimulation of T cells will represent a very small percentage of the total messenger RNA population in induced cells, these gene sequences can be identified by differential hybridization to ^{32}P-labeled cDNA probes from PHA-stimulated and control mRNA populations.

Large quantities of mitogen-induced and noninduced RNA have been isolated from human PBL, obtained by leukophoresis. T cells have been isolated by ficoll hypaque gradient centrifugation and subsequent nylon wool purification.

Following in vitro stimulation, RNA has been purified from such T cell populations and has been demonstrated to be representative of an activated population by the characteristic expression of inducible oncogene mRNA. A cDNA library in the λgt 10 cloning vector has been constructed from a four hour PHA-stimulated mRNA population. Four unique clones have been selected based upon selective hybridization to PHA-induced cDNA probes. ^{32}P-labeled DNA probes were made from these phage inserts by nick translation and hybridized to Northern blots of RNA from unstimulated cells in addition to 1.5 hr and 4 hr PHA stimulated cells. Two of the four clones gave a 2-3 fold stronger hybridization signal on the stimulated RNA than the control RNA. Three of the four clones were subcloned into a plasmid vector and analyzed by nuclear run-on analyses. Each of the three clones demonstrated a 2-3 fold enhancement in specific transcription of its corresponding gene in PHA stimulated human PBL.

To improve the detection of rare clones (and to reduce the size of the library to be screened), we have begun the construction of a subtracted cDNA library. A single stranded cDNA copy of the "stimulated" RNA is hybridized to completion with poly A$^+$ RNA from the unstimulated cells. The RNA-DNA hybrids are removed by hydroxyapatite chromatography and the remaining single stranded cDNA molecules are cloned by standard methods. At present, the construction of such a library has been completed.

Significance to Biomedical Research and the Program of the Institute: Essential to an understanding of the immune response are the intracellular biochemical events which result from antigen and lymphokine binding. We are utilizing an approach based on recombinant DNA technology to define a critically important consequence of lymphocyte activation, the identity of transcriptionally-activated gene sequences. In addition, the physiological mechanisms which initiate and maintain proliferation in lymphocytes are fundamental to the field of growth regulation and cancer biology. Recent advances in this field include the identification of gene sequences, oncogenes, whose increased expression or unregulated expression override normal cellular growth control mechanisms, leading to malignant transformation. By understanding the normal biochemical events associated with proliferation in lymphocytes, one should gain insight into the regulation and cell cycle associated role of oncogenes. Finally, defining regulatory mechanisms underlying the expression of genes which are involved in differentiated function is of central significance to an understanding of gene regulation and development in general.

Proposed Course of Project: 1) Following the definition of tissue-specific (β chain) or growth-specific (c-myc) DNA regulatory sequences, the broad direction of such projects then will concern the characterization of trans-activating factors that regulate gene transcription through recognition of such sequences. 2) A subtracted cDNA library construction is in progress. Upon completion of the library, subsequent selection, isolation, and characterization of positive clones will proceed by standard procedures.

Publications:

Kelly, K.: Growth factors short-circuited by oncogenes. Nature 317:390, 1985.

Kelly, K. and Siebenlist, U.: The expression and regulation of the c-myc gene in normal and malignant cells. Annual Reviews of Immunology 4:317-338, 1986.

Kelly, K. and Underwood, B.: The role of ionic signals in early gene induction during T cell activation. In Oncogenes and Growth Factors 4, G. Van deWoude and T Papas (eds), Elsevier Press, 1986 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05122-02 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

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

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

PI:	A. Rosenberg	Medical Staff Fellow	IB, NCI
Others:	A. Singer	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NIC, NIH, Bethesda, Maryland 20892

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.)*

This project was undertaken to delineate the roles of phenotypically distinct T cell subpopulations in mediating tissue allograft rejection. We demonstrated that $Lyt2^+$ T cells could reject class I but not class II disparate grafts and that $L3T4^+$ T cells could reject class II but not class I disparate grafts. We subsequently investigated the ability of these distinct populations to reject skin grafts bearing (1), multiple minor-H disparities, and (2), a single minor-H disparity, the male specific H-Y antigen. Whereas both untreated and $L3T4^+$ T cell populations can reject grafts bearing multiple minor-H disparities, it is not yet clear if the $Lyt2^+$ population can do so in isolation. In the case of the rejection of H-Y disparate grafts, it is clear that neither purified population in isolation was able to effect the rejection of these grafts and that collaboration between T cell subpopulations was essential for rejection. Additional studies have investigated the ability to effect rejection of grafts by the addition of "carrier" determinants of varying disparities. For example, in a preliminary assay, the swift rejection of bm6 grafts (ordinarily poorly rejected by B6 mice) is accomplished by the addition of a class II disparity (bm12 x bm6) but not by a class I disparity (bm1 x bm6), suggesting that there may be a difference in the ability of helper cells of differing phenotypes to collaborate in effecting the rejection of allografts. By performing these assays using the adoptive transfer model, we hope to delineate the phenotypes of the cells required for such collaborative interactions.

Project Description

Objectives: To study the roles of isolated T cell subpopulations in mediating graft rejection.

To delineate the T cell populations participating in collaborative interactions which lead to the rejection of allograft of varying disparities.

Methods Employed: We utilized an adoptive transfer model consisting of athymic (nude) mice skin grafted and then injected with variously treated cell populations. Specifically, skin grafts (either syngeneic, minor different, class I only or class II only different) were placed on day 1 and the following day mice were injected with $50x10^6$ spleen cells from normal B6 animals that had been either L3T4 + C' treated, Lyt2 + C' treated or untreated. The grafts were assessed daily.

Experiments with normal mice consisted of engrafting B6 mice with tailskin grafts from bm6 animals and either a bm6 x bm12 graft or a bm6 x bm1 graft. The grafts were placed on opposing flanks.

Major Findings: $Lyt2^+$ T cells are both necessary and sufficient to effect the rejection of class I disparate K^b mutant skin grafts, whereas $L3T4^+$ T cells specific for the graft seem to play no role. On the other hand, $L3T4^+$ T cells are necessary and sufficient to effect the rejection of class II disparate grafts. Rejection of H-Y disparate skin grafts is contingent upon the collaborative interaction of $Lyt2^+$ T cells and $L3T4^+$ T cells.

Significance to Biomedical Research and the Program of the Institute: Elucidating the cellular mechanisms and subpopulations responsible for mediating the rejection of tissue grafts bearing various disparities is the critical step in being able to subsequently manipulate them.

Proposed Course of Project: To further elucidate the role of both $Lyt2^+$ and $L3T4^+$ cells in the rejection of allografts.

Publications:

Rosenberg, A. S., Mizouchi, T., and Singer, A.: 1986. Analysis of T cell subsets in the rejection of MHC class I disparate K^{bm} mutant skin allografts. Nature, in press.

Caughman, S. W., Sharrow, S. O., Shimada, S., Stephany, D. S., Mizuochi, T., Rosenberg, A. S., Katz, S. I., and Singer, A.: 1986. Ia^+ murine Langerhans cells are deficient in surface expression of class I MHC. 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 05123-02 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Isolation and Characterization of a Novel Class I MHC Gene

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

PI:	D. S. Singer	Senior Investigator	IB, NCI
Others:	R. Ehrlich	Visiting Fellow	IB, NCI
	D. Margulies	Senior Investigator	LI, AID
	J. McCluskey	Visiting Fellow	LI, AID
	D. Sachs	Branch Chief	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

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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.)*

In all species, the class I MHC genes constitute a family of homologous DNA sequences, some of which encode the heavy chain moiety of the classical transplantation antigens. The functions of the remaining class I genes and their products are poorly, or not at all, understood. In the miniature swine, which contains only 7 class I homologous DNA sequences, two closely related genes encoding classical transplantation antigens have been isolated and characterized. A third sequence, which is highly divergent, has now also been isolated and is being characterized. By molecular hybridization to genomic DNA and direct DNA sequence analysis, it has been demonstrated that this gene, PD6, is a member of the limited class I SLA family. Despite extensive divergence, it appears to share the over-all sequence organization of a class I gene. The PD6 gene is expressed both in transfected mouse L cells and in vivo.

Project Description

Objectives: This laboratory has undertaken to study the members of the class I MHC gene family in the miniature swine, with the aim of understanding their organization and function. Unlike other species such as mouse and man whose class I gene families number 20-40 members, the pig appears to have a restricted family of only 7 members. Of these, 3-5 appear to be closely related, whereas the remainder are more distantly related. The closely related members contain those genes encoding classical transplantation antigens. The purposes of the present studies were to determine: 1) whether the distantly related DNA sequences were indeed members of the class I gene family or of another family and 2) whether these divergent sequences could be expressed and if so the nature of their products. In the mouse, the antigens Qa and Tl, thought to be differentiation antigens, are encoded by members of the class I gene family. Similar differentiation antigen-encoding genes have not yet been characterized in the pig and might be expected to be found among the more distantly related sequences. Such analyses will reveal the genomic organization of the class I family and the maximum number of class I genes necessary for a normal immune response, as well as the functional spectrum of class I-encoded products.

Methods Employed: The clone, PD6, which contains a divergent class I SLA DNA sequence, was isolated from a phage genomic library by cross-hybridization with another SLA gene. The class I-homologous DNA sequences have been characterized by standard techniques: restriction enzyme mapping, molecular hybridization with another SLA gene and sub-genic fragments, and direct DNA sequence analysis (using the M13/dideoxy method of Sanger). Expression of the PD6 sequences has been assessed in both mouse L cells following DNAmediated gene transfer and in vivo by direct filter hybridization of the gene to RNA derived from various tissues or by S1 nuclease analyses of the same RNA samples. Identification of the PD6-encoded product was attempted by two approaches to eliciting PD6-specific antibodies: 1) immunization of mice with PD6-transfected L cells or 2) immunization of mice with L cells transfected with a hybrid gene composed of 5' end PD6 sequences attached to 3' end H2 L^d sequences.

Major Findings: The recombinant clone, PD6, contains an SLA-homologous DNA segment which is highly divergent from other class I DNA sequences. However, it is a member of this multigene family, and not of another distantly related family. DNA sequence analysis and direct hybridization have indicated that PD6 has a sequence organization similar to other class I genes. Analysis of the 5' end of the gene revealed homology in both the 5' flanking region and first exon. A conserved region around the 'CAT' box was observed, but no 'TATA' box was found. Overall homology between PD6 and PD1 is 60%. Open reading frames have been found in all exons, consistent with the possibility that PD6 encodes a functional molecule. Preliminary data suggest a PD6 product has been identified on the surface of transfected mouse L cells.

PD6 is expressed both in transfected mouse L cells and in vivo, as assessed by the presence of PD6-specific RNA. In vivo, PD6 RNA is found in a variety of lymphoid and non-lymphoid tissues, with the highest levels in spleen, lymph node and liver. Among lymphocytes, PD6 is expressed primarily in T cells.

Significance to Biomedical Research and the Program of the Institute: Studies in a number of mammals, including man, mouse and pig have demonstrated the importance of products of some class I MHC genes in normal immune responses, including control of graft rejection and cellular immunity to virus. The role of the remaining members of the class I MHC gene family is not known. The present studies will help define the functions, if any, of these gene products and should establish the minimum genetic requirements for a normal immune response. In addition, comparison of the DNA sequences of the class I MHC genes will provide information about the evolution and generation of diversity of this multigene family.

Proposed Course of Project: Studies on the PD6 class I MHC gene will proceed along the following lines: The nature of the product of the PD6 gene will be investigated using the antisera generated against the transfected L cells. The cellular distribution of PD6 product on various tissues will be determined. In addition, immunoprecipitation studies will reveal the biochemical nature of the PD6 product as well as its association with other polypeptide chains.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05124-02 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Expression and Function of a Porcine Class I MHC Gene in Transgenic Mice

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

PI:	D. Singer	Senior Investigator	IB, NCI
Others:	J. Bluestone	Laboratory Leader	IB, NCI
	R. Hodes	Chief, Immunotherapy Section	IB, NCI
	W. Frels	Agricultural Research Service	USDA

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A porcine class I major histocompatibility complex (SLA) gene has been introduced into the genome of a C57BL/10 mouse. This transgenic mouse expressed SLA antigen on its cell surfaces and transmitted the gene to off-spring, in which the gene is also expressed. Skin grafts of such transgenic mice were rejected by normal C57BL/10 mice, suggesting that the foreign SLA antigen expressed in the transgenic mice is recognized as a functional transplantation antigen. The cellular basis for this recognition is under investigation.

Project Description

Objectives: The introduction of isolated class I MHC genes into cultured mammalian cells has been used to study their expression, antigenicity and antigen-presenting capacity. This approach allows the introduction of genes not normally expressed in the recipient cells, such as foreign, mutant or hybrid MHC genes constructed in vitro. In addition, the expression of individual members of a multigene family can be studied in isolation. However, such studies have been generally limited to a small number of cell types, such as L cells, and the questions addressed have been limited to those related to the expression of the introduced gene in that particular cellular environment. It has not been possible to address developmental issues such as the patterns of tissue-specific expression of individual class I MHC gene or the influence of newly introduced genes or the induction of self tolerance and the maturation of the immune response. To study these unanswered questions, a cloned class I SLA gene was microinjected into the fertilized eggs of mice.

Methods Employed: Male pronuclei of one-cell C57BL/10 (B10) fertilized eggs were injected either with approximately 500 copies of a 9 kb Hind III fragment derived directly from a genomic SLA clone, PD1, or with about 2000 copies of a 5.6 Kb Hind III-Bam HI fragment, subcloned into pBR322 and linearized with either Hind III or Eco RI. These fragments of swine DNA each contain the entire SLA gene. To test for swine DNA sequences, DNA was extracted from a segment of tail of each of the mice and hybridized with an SLA DNA probe. Expression of SLA antigen on the cell surface was assessed by cell-surface labeling of peripheral blood lymphocytes (PBL) with a monoclonal antibody specific for SLA determinants, and analysis by flow microfluorometry.

Major Findings: Of 171 eggs injected with the 5.6 kb Hind III-Bam HI DNA fragment, 100 were transferred to pseudopregnant females, and there were 63 off-spring. None were positive for the SLA gene. Twenty-seven eggs were injected with the 9 kb Hind III DNA fragment and 20 were transferred to a single foster mother; there were 5 off-spring. A single SLA-positive mouse was identified. PBL from this animal were shown to express SLA antigen on their cell surfaces. In an effort to examine the stability of expression and heritability of the SLA gene and to estimate the number of loci into which the SLA gene integrated, this animal was mated to B10 females. All off-spring which contained the SLA gene expressed SLA antigen on their cell surfaces. It was estimated that there is only one, or a few copies, of the SLA gene in the genome of these mice. It is concluded that the SLA gene is in a single integration site and is transmitted to progeny in accordance with Mendelian inheritance. SLA expression in transgenic mice may be regulated: different levels of SLA antigen expression are observed in spleen, lymph node, bone marrow and thymus. The ability of the SLA antigen to be recognized as a transplantation antigen was assessed by engrafting normal B10 animals with tail skin from transgenic and normal B10 mice. All recipients rejected skin from transgenic mice, but not skin from normal mice.

Significance to Biomedical Research and the Program of the Institute: Genetic and cellular studies in a number of mammals, including man, mouse, and pig, have demonstrated that class I MHC antigens play an integral role in the cellular interaction leading to both humoral and cellular immunity. In

particular, the ability to accept tissue grafts appears to be determined by the class I MHC antigens. The ability to study the mechanisms regulating graft rejection and transplantation have been limited by the availability of appropriate pairs differing in only a single gene. The approach described here affords the possibility to study in vivo the effects of isolated genes on transplant rejection. An understanding of the genetic basis underlying transplantation rejection should allow for the development of new therapeutic modalities.

Proposed Course of Project: It should now be possible to determine the relation between those T cells specific for xenogeneic MHC products and the T cells specific for syngeneic and allogeneic murine MHC determinants. Specifically, the ability to generate CTL directed against the SLA antigen will be determined, as well as their precursor frequency and fine specificity, as determined using a panel of allogeneic and mutant cells. The mechanisms mediating murine self-tolerance to the normally foreign SLA antigens can be compared with those involved in conventional self-tolerance to murine H-2 products. Finally it will be of interest to determine the degree to which the murine T cell repertoire can utilize a transfected SLA gene product as a restricting element for antigen recognition and physiologic cell interactions.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09200-01 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Production of Mab Specific for B Lymphocyte Receptors for Lymphokines

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

PI:	M. Principato	Postdoctoral Fellow	IB, NCI
Other:	H. B. Dickler	Senior Investigator	IB, NCI
	E. Park	Postdoctoral Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.25	2.25	

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.)*

The goal of this project is to identify and characterize B lymphocyte receptors for lymphokines via the production of monoclonal antibodies. One promising Mab (F1-10) has been obtained so far. This antibody binds to LPS stimulated B lymphoblasts and some B cell tumors. Minimal binding is seen with a minority of normal B cells and bone marrow cells. Peripheral T lymphocytes, thymocytes, and concanavalin A stimulated T lymphoblasts are negative. Time course experiments reveal that expression of the F1-10 antigen peaks 60 hours after stimulation with LPS and the kinetics of expression is distinct from other B lymphocyte membrane molecules. Strain distribution studies reveal that expression of the F1-10 antigen is linked to H-2 in that DBA/2 (prototypic strain) and B10.D2 are positive while B10 is negative. However, control of expression is complex in that other strains which are haplotype identical to DBA/2 in one or several of the H-2 regions (K,I,D,T1A) are negative. These data suggest that F1-10 is recognizing a previously undescribed B lymphocyte membrane molecule and are consistent with the possibility that this molecule is a lymphokine receptor.

GPO 914-918

Project Description

Objectives: Identify and characterize B lymphocyte membrane molecules which are receptors for growth and differentiation inducing lymphokines.

Major Findings: A rat IgG2b,k mAb has been obtained which binds to LPS stimulated B lymphoblasts and certain B cell tumors. A minority of normal spleen B cells (10-20%, the larger B cells as assessed by light scatter) are weakly positive and 10-20% of bone marrow cells are also weakly positive. Thymocytes, normal T cells from spleen, and concanavalin A 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.

Expression of the Fl-10 antigen appears to be linked to the H-2 complex in that DBA/2 (prototypic strain) and B10.D2 are positive while B10 is negative. However, other strains which are haplotype identical to DBA/2 in one or several of the regions of the H-2 complex (K,I,D,Tla) are negative including Balb/c, B10.GD, B10.HTG, B10.A, A/J, A.AL and A.D2. Thus, the control of expression is complex. The major possibilities are that expression is controlled by a gene mapping to the left of K or the right of Tla, or that a background gene(s) also plays a role in expression of the Fl-10 antigen. Interestingly, strains with lymphoproliferative or autoimmune defects (AKR, NZB, MRL) are also Fl-10 positive.

Taken together, the tissue distribution, kinetics and genetics of Fl-10 antigen expression suggest that this is a previously undescribed B lymphocyte membrane molecule. The data are consistent with the possibility that this molecule is a lymphokine receptor.

Proposed Course of Project: 1) Further characterization of the Fl-10 molecule including quantitation and genetics of expression, size and chemical nature and functional role in B lymphocyte responses and/or disease states; 2) production of mAb specific for other lymphokine receptors.

Publications: None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09201-01 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Interaction of B Lymphocyte Subpopulations

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

PI:	F. Uher	Postdoctoral Fellow	IB, NCI
Other:	H. B. Dickler	Senior Investigator	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

The goal of this project is to characterize interactions between B lymphocyte subpopulations which regulate responses of these cells. Previous work from this laboratory showed that a B lymphocyte hybridoma (2.4G2) produced a low molecular weight substance(s) which triggered B lymphocytes to both proliferate and secrete antibody. This result suggested that certain B lymphocytes might regulate the response of other B cells. Our current studies have shown 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 $F(ab')_2$ 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

Objectives: Characterization of the interactions between B lymphocytes which regulate humoral immune responses.

Major Findings: The hybridoma 2.4G2 but not the parental fusion partner P3U1 produces a low molecular weight factor which stimulates proliferation and antibody secretion from B lymphocytes in the absence of T lymphocytes. Responses to this factor are polyclonal and mainly of the IgM class. Responses were obtained primarily from larger B cells which bear the LyB5 alloantigen. Adherent accessory cells were not required for these responses. These results suggest that some B cells may produce factor(s) which promote growth and/or differentiation of B lymphocytes.

Purified (96% sIg positive, 0.1% T cells, 0.1% macrophages) small (120 μ^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 responder 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 augmentation 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 augmentation 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.

Proposed Course of Project: Due to current lack of personnel, the only immediate plans are to publish our current results. When personnel become available our plans include: 1) characterization of the soluble and membrane components of augmenting activity; 2) evaluation of the effects of this activity on hypo-responsive B cells from neonates and immunodeficiency states; 3) evaluation of whether the augmenting activity of activated B cells could be used to culture long-term normal B cell lines.

Publications: None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09202-01 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Characterization of T Cell Receptor Genes in Alloreactive Clones

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

PI:	Hana Golding	Fogarty Visiting Scientist	IB, NCI
Others:	Dinah Singer	Senior Investigator	IB, NCI
	William Biddison	Senior Investigator	NI, N

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

A panel of alloreactive T cell clones derived from a single donor has been established and characterized for its fine specificity patterns. It has thus been possible to assign each of the clones to one of four fine specificity groups. In order to attempt to correlate fine specificity with receptor gene utilization, the TcR alpha and beta variable gene segments used by these clones are being analyzed. An alpha variable gene derived from a member of the most common group is being isolated from a restricted cDNA library. Its representation in the other members of the panel will be assessed.

Project Description

Objectives: The objective of this study is to attempt to assess at a molecular level the relationship between receptor fine specificity and variable gene usage. The receptor repertoire of T cells is derived from a multiplicity of variable genes encoding both the alpha and beta chain components of the receptor. The rules governing the utilization of these genes is not understood. Therefore, it is of interest to examine a panel of clones, derived from a single donor, directed at a common alloantigenic specificity, to determine to what extent the variable genes used are distinct or in common.

Methods Employed: A restricted cDNA library was constructed from one of the clones using standard techniques for first and second strand synthesis, tailing, annealing and transfecting. Clones are currently being screened for the presence of alpha cDNA inserts using a constant region probe derived from the constant region gene of the murine alpha chain.

Major Findings: Using an oligonucleotide derived from the constant region domain of the alpha chain gene, it has been possible to construct a restricted cDNA library which should be highly enriched for the presence of alpha cDNA clones. These clones are currently being screened, using a probe distinct from the primer.

Significance to Biomedical Research and the Program of the Institute: The T cell receptor repertoire consists of the diversity of alpha and beta variable genes, which combine to generate the T cell receptor. Since this receptor is vital to generating both cellular and humoral immunity, understanding the molecular basis for generating receptors with common antigens, but distinct fine specificities, should help to elucidate the mechanisms which regulate receptor generation.

Proposed Course of Project: Clones containing the alpha chain gene will be isolated and characterized. Their representation in the remaining T cell clones of the panel will be assessed.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09203-01 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Isolation and Characterization of a Novel H2 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
	J. Hare	Summer Guest Worker	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.75	.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 highly divergent member of the class I MHC gene family has been isolated from the genome of the C57BL/10 mouse. This gene, Mbl is linked to the remaining members of the family, mapping to the D-end of the complex. Although Mbl is not expressed in transfected mouse L cells, it is expressed in vivo in a variety of tissues, with preferential expression in T cells. Furthermore, it is expressed in high levels in a T cell lymphoma. DNA sequence analysis reveals that the exon organization of this gene resembles that of other class I genes. However, the level of DNA sequence hemology of Mbl to other H2 genes is no greater than to human HLA genes. Furthermore, whereas all previously reported H2 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.

PHS 6040 (Rev. 1/84)

Project Description

Objectives: 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, and potentially more, members of the family which is highly divergent from the rest of the class I genes of the mouse. 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.

Methods Employed: Genomic libraries were constructed in the bacteriophage vector Charon 4A and screened with a human cDNA probe at low stringency. One hybridizing clone was selected for further analyses. Following restriction enzyme mapping to localize the gene within the clone, DNA sequence analysis of the gene was performed using either: 1) standard M13/dideoxy sequencing, deletion sequencing, or oligonucleotide primed sequencing. Mapping of the gene within the H2 was by Southern hybridization to genomic DNA from a variety of wild and inbred mouse strains. Expression of Mbl was by Northern analysis of RNA derived from a variety of mouse tissues, transfected L cell lines, and mouse tumors.

Major Findings: The MBl gene is a previously undescribed gene which maps to the D-end of the H2 complex. DNA sequence analysis demonstrates that Mbl is no more related to H2 class I genes than to either human or pig. Furthermore, the over-all organization of the gene is more similar to human than to mouse. These data suggest that Mbl may be a descendant of an ancestral gene which predated speciation. Analysis of RNA samples derived from a variety of tissues demonstrates that Mbl is expressed in vivo, in a regulated fashion.

Significance to Biomedical Research and the Program of the Institute: Products of the MHC play a pivotal role in cellular interactions leading to immune responses. Class I MHC gene products have been demonstrated to act as viral restriction elements. The existence of a highly divergent class I MHC gene, which is expressed in vivo raises the possibility that it has been retained in evolution because its product may have important functions in the immune response. Therefore, analysis of this gene should shed light not only on the evolution of the multigene family, and the mechanisms regulating it, but also on the function of its product.

Proposed Course of Project: DNA sequence analysis of the gene will be completed to ascertain whether MBl is capable of encoding a functional product. In conjunction with this, studies are currently in progress to isolate cDNA clones of Mbl, which will then be introduced into expression vectors in order to generate large amounts of product for analysis.

Publications: None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09204-01 I

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Function of Accessory Molecules in T Cell Interactions

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

PI: S. McCarthy — Staff Fellow — IB, NCI

Other: A. Singer — Senior Investigator — IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	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.)*

Precursor and effector CTL specific for determinants present on allogeneic MHC class I molecules have previously been shown to bear the Lyt2 cell surface phenotypic marker. Lyt2 is believed to be involved in the function of these cells because antibodies directed against Lyt2 block the cytolytic activity of most in vitro-generated MHC class I allospecific CTL. Based on those studies, Lyt2 has been proposed to be either an accessory molecule contributing to antigen specificity, or an "off" signal. Our studies have focused on a novel population of class I-specific CTL, which are induced in vitro in the presence of anti-Lyt2 monoclonal antibody and are refractory to functional blocking by subsequent exposure to anty-Lyt2 antibody. These novel CTL are $Lyt2^+$ and adsorb surface anti-Lyt2 monoclonal antibody during culture. However, unlike conventional $Lyt2^+$ CTL, they exhibit selective resistance to lysis with anti-Lyt2 antibody and complement. Since CTL with this unusual characteristic are generated only in the presence of anti-Lyt2 antibody, Lyt2 may serve as a positive signal transducing molecule on the surface of some T cells. Thus, Lyt2 may act as on "on" signal during CTL differentiation.

Project Description

Objectives: The primary objective of this project is to characterize the function of Lyt2 molecules on the surface of allospecific cytolytic T lymphocyte (CTL) precursor and mature effector cells.

Methods Employed: Primary in vitro CTL responses were induced in mixed leukocyte cultures (MLC) of spleen cells from H-2 congenic mouse strains. CTL activity generated during culture was assayed by lysis of ^{51}Cr-labeled activated target cells. Stable long-term CTL lines were established by weekly restimulation of CTL generated in vitro. CTL were characterized by negative selection with antibody plus complement treatment and by fluorescence staining and flow microfluorometry.

Major Findings: In contrast to conventional $Lyt2^+$ class I allospecific CTL, CTL generated against class I MHC alloantigens in the presence of anti-Lyt2 monoclonal antibody (mAb) are resistant to blocking of their lytic effector function by additional exposure to anti-Lyt2 mAb. Limiting dilution frequency analyses (LDA) indicate that anti-Lyt2 mAb in the MLC actively induces development of these novel, anti-Lyt2 resistant CTL, which develop only rarely in conventional LDA cultures lacking the anti-Lyt2 mAb. These CTL express surface Lyt2, efficiently bind anti-Lyt2 mAb, and can be lysed with anti-Lyt2 mAb and rabbit complement. They exhibit, however, a unique resistance to lysis with anti-Lyt2 mAb and guinea pig complement, which effectively lysis conventional $Lyt2^+$ class allospecific CTL. We have shown that functionally modified Lyt2 surface expression on these novel CTL is induced very early in culture and depends upon specific antigen stimulation as well as exposure to anti-Lyt2 mAb. We have derived long-term stable CTL cell lines with these same novel characteristics, which will facilitate biochemical and genetic analyses of the nature and function of Lyt2 molecules on CTL.

Significance to Biomedical Research and the Program of the Institute: Characterization of the signals and signalling molecules involved in the induction of allospecific CTL is necessary in the development of strategies for regulating CTL activity against malignancies and allogeneic tissue grafts.

Proposed Course of Project: We plan to use long-term novel CTL cell lines to analyze the signal capacities of cell surface Lyt2 molecules, and to extend our studies to $Lyt2^+$ MHC class II-specific CTL.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09205-01 I

PERIOD COVERED

October 1, 1985 to September 30, 1986

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	IB, NCI
Others:	K. S. Hathcock	Chemist	IB, NCI
	D. M. Segal	Senior Investigator	IB, NCI
	M. Taplits	Medical Staff Fellow	IB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

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 standard 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.

Project Description

Objectives: The structures and mechanisms involved in triggering of T cells through cell surface receptor structures are to be studied.

Methods Employed: Assays of T cell proliferation were carried out through standard techniques employing measurement of ^{3}H-thymidine. Generation of cloned T cell populations and heterogeneous T cell subpopulations was performed through methods previously described. Preparation of antibody heteroaggregates was described in Project No. Z01 CB 05050-12 I.

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, F23.1 covalently linked to anti-A^{k}_{α} A^{k}_{β} antibody was capable of activating Lyt2 or $L3T4^{+}$ T cells in the presence of IL-2 and A^{k}_{α} A^{k}_{β}-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.

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.

Significance to Biomedical Research and the Program of the Institute: T cell responses play an apparent/critical role in host responses to malignant cells and infectious agents. The basic mechanisms of T cell recognition and activation are critical to an understanding and potential manipulation of such responses. The present studies are therefore being directed at the structural and mechanistic processes involved in receptor mediated T cell activation.

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.

Publications:

None

Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1985 to September 30, 1986

During the last year, the Laboratory of Immunobiology underwent two major changes: the elimination of the Humoral Immunity Section (its actvities were merged with that of the Office of the Chief) and the departure of Dr. S. H. Ohanian; as a consequence of his leaving, research into the mechanism of killing of nucleated cells by antibody and complement and the mechanism of resistance to such killing ceased. The remaining members of the Laboratory of Immunobiology continued investigating the effector arm of the immune system: in the Office of the Chief, analysis of the interaction of antibodies, antigens and complement components continued; in the Immunopathology Section studies focussed on chemotaxis and inflammation; and the central theme of the Cellular Immunity Section was the immunologic and genetic bases of tumor rejection in vivo. Close collaboration among the members of the Laboratory continued at a high level.

In the Office of the Chief three areas of the interaction of antibodies, antigens and complement were under study. In the first, analysis of the dissociation of rabbit anti-methotrexate (MTX) IgG from cell bound MTX under various conditions was continued (in collaboration with Dr. R. Mage, NIAID). Dissociation was quantitated with fluid phase hapten preventing reassociation of the anti-hapten antibodies. More than 90% of the anti-MTX IgGs was prevented from reassociation by nanogram quantities of fluid phase MTX. In contrast, no dissociation of some anti-MTX IgG-anti-IgG complexes could be measured even at 24 hrs of incubation in the presence of as much as 100 mg fluid phase MTX. Crosslinking of anti-MTX IgGs by the anti-IgG antibody was excluded by performing the experiments in large excess of anti-IgG antibody, by showing the phenomenon was independent of anti-MTX antibody density, by showing that decreased dissociation was temperature independent and by showing that aggregation induced by protein A induced decreased dissociability albeit by three orders of magnitude lower than the anti-IgG antibody. The extent of reduction of dissociability was a function of the anti-IgG antibody. Most effective were rabbit anti-b4 allotype antibodies while some anti-b5 antibodies were not as effective. This latter observation supports the notion that there may be different b5 allotyopes in rabbits. Anti-a1 was also less effective than anti-b4; The results suggested that the anti-IgG antibody reduced the mobility of the Fab arms of the anti-hapten antibody, thereby effectively locking the arms in a rigid position. Locking the arms reduces the chance of fluid phase hapten to compete with the cell surface hapten for the antigen binding site in the Fab arm which is equivalent to reducing dissociability of the anti-hapten IgG. One implication of this observation is that a highly dissociable auto-antibody at a cell surface may induce only limited damage; an immune complex which contains antibody to the auto-antibody may be damage producing because it is a large, non-dissociable complex.

In the second, binding and activity of C1q (the antibody recognizing subcomponent of C1) was studied. In collaboration with the Lausanne group (Professor Henri Isliker, principal collaborator) we found that fibronectin, a molecule that reacts with collagen inhibited the interaction of C1q with IgG if the interaction between fibronectin and C1q occurred in the fluid phase before exposure to the IgG. Once C1q bound to the antibody, fibronectin could not inhibit C1q activity. This observation supported our contention that the physical form of bound C1q is different from that of free C1q. We are investigating this question by studying the binding of C1q to IgG and IgM antibodies at cell surfaces in the presence and absence of C1r and C1s, the other subcomponents of C1. We predicted that fluid phase C1q should interact with C1r-C1s in a different manner than cell bound C1q. Preliminary observations tend to confirm this prediction. This is an important question for it implies that generation of effective C1 cannot occur once C1q is bound.

The third area we studied was the influence of suramin on the generation of the complement mediated cell lesion. We have shown many years ago that the generation of the cell membrane lesion by C8 and C9 (the components that penetrate the cell membrane and form a channel that permits the passage of low molecular weight substances) is a multistep process; that among the steps were the possible dimerization of C8 and polymerization of C9. We could distinguish several fuctional steps of the C9 reaction, which were binding, insertion, channel formation and passage of low molecular weight substances. Suramin interferes with insertion and channel formation; the interference is reversible. This observation furnishes another tool to study the formation of the C channel.

The Immunopathology Section has continued studies on chemotaxis and inflammation. It was established last year that although fMet-Leu-Phe is thought to be the prototype of chemoattractants produced by bacteria, virtually all the chemotactic activity in filtrates of S. aureus is due to peptides other than fMet-Leu-Phe. The efficacy (number of monocytes responding) of the bacterial peptide attractants exceeds that of fMet-Leu-Phe. Structural similarity to fMet-Leu-Phe is suggested by the finding that partially purified bacterial peptides inhibit binding of fMet-Leu-Phe-Lys-FITC to human monocytes. The objective this year was to purify and determine the structure of the most active peptide. A single peak of chemotactic activity was obtained by sequential purification by reverse phase HPLC on C-18 and diphenyl columns. The amount of peptide in this peak was below the detection limit of the most sensitive amino acid analysis, indicating that the peptide is effective as an attractant at 10^{-10}M or less. Since no structural data will be forthcoming without a greatly increased amount of starting material, production of bacterial culture fluid has been increased from 1 liter to 75 liters, in preparation for large scale purification.

An IgG monocloncal antibody to human macrophage stimulating protein (MSP) was obtained last year. A column of IgG anti-MSP covalently linked to Sepharose was used for a single step purification of MSP from human plasma; the pH 2.5 glycine buffer eluate had high activity in the standard bioassay (enhancement of peritoneal macrophage responsiveness to chemoattractant). SDS polyacrylamide gel electrophoresis under reducing conditions, with transfer to nitrocellulose paper and development with monoclonal mouse anti-MSP, showed that the monoclonal antibody

column purified MSP fraction contained at least 5 detectable proteins. Thus, either the monoclonal anti-MSP was directed against a common epitope on 5 different plasma proteins, or the antibody preparation was not the product of a single clone. The putative clone was recloned; the 5-protein Western blot pattern developed with IgG of two different clones from the recloning was identical to that of the original clone. Since immunoassay of MSP blood levels and determination of MSP tissue localization require an antibody that detects only MSP, another set of spleen cell fusions from mice with anti-MSP activity is now planned.

The interaction of leukocytes with the chemoattractant fMet-Leu-Phe results not only in directed movement but also in a metabolic burst that is associated with the generation of toxic oxygen radicals. It was shown that the burst limits the magnitude of the neutrophil chemotactic response because of oxidation of the attractant and diminution in the chemotactic gradient. The number of neutrophils migrating to fMet-Leu-Phe over the whole dose-response range increased from about 40% of the input number to 70% when agents that prevented oxidation of the attractant were included in the assay. These included mercaptoethanol, catalase and low concentrations of albumin. On the other hand, these agents had no effect on the response to fNle-Leu-Phe-Nle-Tyr-Lys, a chemotactic peptide without oxidizable amino acids, showing that the protective action was on the attractant rather than the responding cells. Other studies on the induction by peptide stimuli of the metabolic burst, which is of 2-3 minutes duration, centered on the responsiveness of human blood monocytes to the stimulus. It was found, in experiments with cold-stored or adenosine-treated monocytes, that a normal response required a responsive cell prior to application of the stimulus. Rapid removal of the metabolic block post-stimulus did not restore the response. The results are consistent with the rapid decay of an intermediate in the stimulus-response pathway, which may also account for the brevity of the metabolic burst. An additional finding of interest was the synergistic effect of fMet-Leu-Phe on adenosine-treated monocytes, which caused an increase in cellular cAMP that exceeded the sum of the effects induced by each agent alone. This is apparently due to the combination of increased adenlyate cyclase caused by adenosine and decreased phosphodiesterase activity caused by fMet-Leu-Phe. The finding may have implications for negative feedback control in other excitable cells.

The Cellular Immunity Section has continued studying the mechanism of the previously reported inhibition of growth of primary rat mammary carcinomas after infusion of plasma absorbed with Protein A-Sepharose or inactivated CNBr Sepharose. The results of our experiments provide evidence for a role of proteins of the alternative pathway of complement in the inhibition of tumor growth. This conclusion was based on correlating the effects of chelating agents on consumption of the first, fourth and third components of complement (C1, C4 and C2) with the effects of these agents on in vivo antitumor activty. A chelating agent (EDTA) which binds both Ca^{++} and Mg^{++} inhibited consumption of the third component of complement and prevented generation of serum factors with in vivo antitumor activity. Another chelating agent (EGTA) which preferentially binds Ca^{++} did not inhibit consumption of the third component of complement and permitted generation of serum factors with in vivo antitumor activity.

The differential effect of these two chelating agents on generation of factor(s) strongly suggests that activation of the alternative pathway in involved in the observed antitumor effects.

Animal models sytems were established for studying the basis of escape of antigenic tumors from host immunological attack. These experiments were based on the following observations. Infection of guinea pig fibrosarcomas with a mouse retrovirus alters the host response to the fibrosarcoma. Fibrosarcomas that grow progressively in syngeneic animals after infection with a mouse retrovirus grow temporarily and then regress. Occasionally tumors reappear at sites of inoculation of retrovirus-infected tumor cells. These tumor recurrences lack viral cell surface antigens, are susceptible to retroviral infection and lack the provirus. Clones were isolated from the retroviral-infected cell population and tested as sources for tumor recurrence. One clone was isolated that produced variants that lacked the provirus but contained a single abbreviated copy of the provirus. Variants were selected in virus-immune guinea pigs. These results provide evidence that gene deletion may be a mechanism by which antigenic tumors may escape host immune attack.

A second example of this phenomenon was provided by studies of a mouse melanoma. Mouse tumor cells (B16-BL6, $H2^b$) were transfected with a gene ($H2^d$) encoding a foreign cell surface antigen. Cloned tumor cells expressing $H2^d$ were injected into immunodeficient mice, normal nonimmune mice or mice immunized to $H2^d$ antigens. Pulmonary metastases from both immune and nonimmune mice (but not immunodeficient mice) had deleted the $H2^d$ gene.

Mouse tumor cells (B16-BL6) were transfected with a gene encoding resistance to the antibiotic G418. Colonies isolated during this procedure contained unique markers that were detectable by analysis of the DNAs extracted from the cells. The utility of these markers were shown by a study of the clonality of pulmonary metastases. Clones containing unique genetic markers were mixed and injected into the footpads of normal syngeneic mice. Although footpads contained both genetic markers, virtually all pulmonary metastases contained single genetic markers. This technique simplifies the technical approach to studies of clonality of metastases and may prove to be widely useful in studies that require accurate identification of tumor cell populations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08525-10 LIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Immunotherapy of Primary Autochthonous Cancer

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

PI:	B. Zbar	Chief, Cellular Immunity Section	LIB NCI
	T. Borsos	Chief, Laboratory of Immunobiology	LIB NCI
OTHER:	K. Nakanishi	Visiting Fellow	LIB NCI
	G. Glenn	Medical Staff Fellow	LIB NCI
	T. Yano	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRF, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The primary objective of this project is to evaluate the therapeutic efficacy of various biologic response modifiers in animals with primary cancer as a guide for treatment of human cancer.

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

Major Findings:

We have continued studying the mechanism of the previously reported inhibition of growth of primary rat mammary carcinomas after infusion of plasma absorbed with Protein A-Sepharose or inactivated CNBr Sepharose. The results of our experiments provide evidence for a role of proteins of the alternative pathway of complement in the inhibition of tumor growth. This conclusion was based on correlating the effects of chelating agents on consumption of the first, fourth and third components of complement (C1, C4 and C2) with the effects of these agents on in vivo antitumor activty. A chelating agent (EDTA) which binds both Ca^{++} and Mg^{++} inhibited consumption of the third component of complement and prevented generation of serum factors with in vivo antitumor activity. Another chelating agent (EGTA) which preferentially binds Ca^{++} did not inhibit consumption of the third component of complement and permitted generation of serum factors with in vivo antitumor activity. The differential effect of these two chelating agents on generation of factor(s) strongly suggests that activation of the alternative pathway in involved in the observed antitumor effects.

Publications:

Nakanishi, K., Zbar, B., and Borsos, T.: Plasma Therapy of Primary Rat Mammary Carcinoma: Dependence of C3 Consumption during Absorption of Plasma with Sepharose Derivatives on the Anticoagulant. Cancer Res., 45:4122-4127, 1985.

Nakanishi, K. Zbar, B., Borsos, T. and Glenn, G. Serotherapy of Primary Rat Mammary Carcinoma: Inhibition by Ethylenedinitrotetraacetic Acid but not by [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid. Cancer Res., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08528-10 LIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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)*

J. Talmadge	Program Resources Inc., NCI-FCRF
B. McEwen	Upjohn Pharmaceutical, Kalamazoo, Michigan

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRF, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	0.4	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard 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.

GPO 914-918

Major Findings:

We investigated the mechanism by which tumors recur at sites of injection of retrovirus-infected fibrosarcoma cell lines. Previously we established that tumor recurrences reflect outgrowth of rare cells that lack viral antigens and are susceptible to superinfection with the homologous retrovirus. In the present study clones isolated from a retrovirus-infected cell line were evaluated as precursors for tumor recurrence. Under conditions of in vivo immunological selection a clone that contained a single abbreviated copy of the provirus formed variants that lacked the proviral gene. Tumor variants lacking the proviral gene grew progressively in both nonimmune and virus-immune animals. Tumor recurrence could be prevented by superinfection of the virus-infected fibrosarcoma cell line or by superinfection of the precursor for tumor recurrence. Cell lines infected with retroviruses varied in frequency of tumor recurrence formation. This model may be useful in analyzing gene deletion as a mechanims of tumor escape from host immunological attack.

The interrelationships between gene expression, host selection and tumor evolution were analyzed by studying the influence of a transfected gene on tumor growth and metastasis and analyzing the effect of host factors on expression of the transfected gene. B16-BL6 cells ($H\text{-}2^b$) transfected with a gene encoding a Class I antigen (D^d) or a plasmid lacking the D^d gene were injected into the footpad of nonimmunized syngeneic mice or syngeneic mice immunized to $H\text{-}2^d$ antigens. Primary tumors were excised when they reached 1.0 cm in diameter. We recorded the median length of time required for primary tumors to reach 1.0 cm in diameter, median survival after tumor excision and the median number of lung metastases. In nonimmunized mice, there were no differences between the growth of primary tumors formed by transfectants expressing D^d and those formed by transfectants lacking D^d; however survival was prolonged after excision of tumors formed by transfectants expressing D^d. In immunized mice, growth of tumors formed by transfectants expressing D^d was significantly retarded and survival after tumor excision was prolonged. The evidence indicates that cell lines established from primary tumors and pulmonary metastases lost their antibiotic resistance and expression of $H\text{-}2^d$ antigen. DNA extracted from local tumors and pulmonary metastases was tested to determine the presence of the transfected plasmid by hybridizing it with ^{32}P-labeled gene probes that detected either the D^d gene or the bacterial plasmid. Pulmonary metastases of immunized and nonimmunized mice and primary tumors of immunized mice had lost the D^d gene and, in most cases, all of the associated plasmid. In contrast, in immunodeficient nude mice, primary tumors and pulmonary metastases retained the D^d gene and the associated plasmid. Deletion of genes encoding cell surface antigens may be a mechanism by which tumors escape immunological attack.

The possible clonal origin of pulmonary metastases from the B16-BL6 melanoma was evaluated using the technique of Southern hybridization. Genetic markers were introduced into B16-BL6 cells by the transfection of a plasmid containing a bacterial gene. Genetic markers were detected by hybridizing DNA from tumors with a ^{32}P-labeled bacterial gene. Clones containing unique genetic markers were mixed and injected into the footpads of normal syngeneic mice. Footpad tumors were amputated when tumors were 1 cm in diameter. Individual pulmonary metastases were harvested and expanded by implantation in nude mice. Although footpad tumors contained both genetic markers, virtually all (13/15) pulmonary metastases contained single unique genetic markers. Two examples of pulmonary metastases of mixed origin were detected. The Southern hybridization technique was sufficiently sensitive to detect a clone that was present at 5% of the total cell population.

Publications:

Tanio, Y., Talmadge, C., Dekaban, G., Hovis, J., Ohanian, S.H., and Zbar, B. In vivo immunologic selection of proviral gene deletion variants from a nonproducer clone. J. Natl. Cancer Inst. in press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08552-20 LIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Mechanism of Complement Fixation and Action

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

PI:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI
OTHER:	A. Circolo	Visiting Associate	LIB NCI
	M. Kirschfink	Visiting Fellow	LIB NCI
	S. Hosoi	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

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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.)*

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.

Major Findings. Binding between a hapten and its antibody is a reversible process in which the effective association constant is the function of many factors in addition to the value of the intrinsic binding constant. In studying the dissociation of cell bound anti-hapten antibody in the presence of an anti-antibody we discovered that in several instances the anti-antibody prevented the dissociation of the anti-hapten antibody from the cell surface. Dissociation was quantitated with fluid phase hapten preventing reassociation of the anti-hapten antibodies. More than 90% of the anti-hapten IgG alone was prevented from reassociation by low concentrations of fluid phase hapten (nano- to microgram ranges). In contrast no dissociation of some IgG-anti-IgG complexes could be measured even in the presence of very large excess of fluid phase hapten (100 mg/ml). Because the conditions of the experiments did not support crosslinking of the anti-hapten IgG by the anti-antibodies, we postulated that the direct interaction of the two antibodies changed the association of the anti-hapten antibody with the hapten. Although the mechanism of the increase in association is unknown, we have postulated that it is the result in the reduction of the mobility of the Fab arms of the anti-hapten antibody by the anti-antibody. The reduction of mobility "locks" the Fab arms in a rigid position thereby reducing the chance of fluid phase hapten to compete with the cell surface hapten for the antigen combining site on the Fab arm. This hypothesis readily explains our findings; tests for validity must await further experimentation.

Lysis of EAC1-8 depends on the formation of a stable transmembrane channel formed by the interaction of C8 in the complex with 1 or more molecules of C9. It has been shown that the generation of the channels is a multistep event proceeding in the order E*(C9 bound)--> E*(C9 inserted) -->E*(C9 doomed)--> ghost. We have found suramin a potent inhibitor of E* transformation. In this study we determined which step was affected by suramin. EAC1-7 were generated with purified C1 and C4-7 reagents; EAC1-8 and EAC1-9 (E*) with purified C8 and C9. E*(C9 inserted) were generated with C9 in the presence of $5x10^{-3}$ M Zn++; human C reagents were used. Suramin inhibited all reactions after EAC1-7 in a dose dependent fashion to below 10 ug/ml. Conversion of E*(C9 bound) to ghost was irreversibly inhibited while that of E*(C9 inserted) was reversibly inhibited. It was concluded that suramin inhibited fluid phase or cell surface bound C9 in an irreversible fashion and that membrane inserted C9 was probably prevented from forming a channel in the presence of the drug.

Binding and activity of C1q (the antibody recognizing subcomponent of C1) was also studied. In collaboration with the Lausanne group (Professor Henri Isliker, principal collaborator) we found that fibronectin, a molecule that reacts with collagen inhibited the interaction of C1q with IgG if the interaction between fibronectin and C1q occurred in the fluid phase before exposure to the IgG. Once C1q bound to the antibody, fibronectin could not inhibit C1q activity. This observation supported our contention that the physical form of bound C1q is different from that of free C1q. We are investigating this question by studying the binding of C1q to IgG and IgM antibodies at cell surfaces in the presence and absence of C1r and C1s, the other subcomponents of C1. We predicted that fluid phase C1q should interact with C1r-C1s in a different manner than cell bound C1q. Preliminary observations tend to confirm this prediction. This is an important question for it implies that generation of effective C1 cannot occur once C1q is bound.

Publications

Kratz, H. J., Borsos, T. and Isliker, H.: Effect of fibronectin on the haemolytic activity of complement. Ann. Inst. Pasteur/Immunol. 136:97-103, 1985.

Borsos, T., Circolo, A., Young-Cooper, G.O. and Mage, R.: Anti-hapten IgG antibodies to cell surface hapten: anti-IgG antibody prevents dissociation as measured with fluid phase hapten. J. Immunol. 136: 224-229, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08577-01 LIB

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Restriction fragment length polymorphisms in normal and neoplastic human 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:	H. Brauch	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

Marston Linehan	Surgery Branch, NCI
Adi Gazdar	Naval Medical Oncology Unit, NCI

LAB/BRANCH
Laboratory of Immunobiology

SECTION
Cellular Immunity Section

INSTITUTE AND LOCATION
NCI-FCRF, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

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

B

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

The primary objective of this project is to compare restriction fragment length polymorphisms (RFLPs) in DNA extracted from normal and neoplastic human tissues. The initial study will focus on renal cell carcinoma and small cell lung cancer. We will look for evidence of deletion of specific chromosomal loci (change in RFLP from heterozygosity to homozygosity).

Major Findings:

Over the past 6 months we extracted DNA from normal and neoplastic tissues of 12 patients with renal cell carcinoma. Gene probes were prepared that detect specific human chromosomal loci.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08575-14 LIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Inflammation

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

PI:	E. Leonard	Chief, Immunopathology Section	LIB NCI
OTHER:	Antal Rot	Visiting Fellow	LIB NCI
	Teizo Yoshimura	Guest Researcher	LIB NCI

COOPERATING UNITS *(if any)*

L. Henderson Litton Bionetics Basic Research Program, FCRF

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Immunopathology Section

INSTITUTE AND LOCATION

NCI, NIH, FCRF, Frederick, MD 21701

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

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.

Major findings: macrophage stimulating protein (MSP) is a mammalian serum protein, discovered in this laboratory, that greatly enhances macrophage motility responses. To develop an immunoassay of human serum MSP, we immunized mice with partially purified MSP and screened fusion product culture fluids. Hybridoma antibody to human MSP was detected by adsorption from culture wells onto protein-A-Sepharose disposable microliter columns, after which MSP was applied to each column and residual MSP in the eluate was measured by bioassay. Depletion of eluate MSP reflected adsorption of MSP by protein-A-Sepharose-anti-MSP. One clone was obtained that produced IgG anti-MSP. A CNBr-Sepharose-anti-MSP column was used to purify MSP from 1 liter of plasma. SDS-PAGE of affinity purified MSP revealed at least 12 bands, from 45 to more than 220 kilodaltons. Specific interaction of monoclonal anti-MSP with 10 of these molecules was confirmed by Western blot. The anti-MSP clone was then re-cloned. IgG from two of the re-cloned colonies produced Western blot patterns identical to that of the original clone. Thus, the antibody interacting with multiple plasma proteins was indeed monoclonal. This illustrates the importance of determining the interaction spectrum of a monoclonal antibody for use in immunoassay of a protein in complex mixtures. When physicochemically isolated MSP was purified on the anti-MSP column, Western blot analysis of the product detected no protein at the expected MSP m.w. of 100 kd. Based on detection sensitivity of the Western blot, MSP concentration in human serum is less than 10^{-10}M.

Products of bacteria are potent chemoattractants for mammalian leukocytes. Several reports suggest that these attractants are small peptides. We compared the properties of culture fluids of Staphylococcus aureus with fMet-Leu-Phe, considered a prototype of bacterial attractant. Virtually all the chemotactic activity in filtrates of S. aureus is due to peptides other than fMet-Leu-Phe. Chemotactic activity for human monocytes of S. aureus culture filtrates was determined in multiwell chemotaxis chambers. At optimal concentrations, the filtrate attracted almost twice as many monocytes as fMet-Leu-Phe (53 $\pm$ 5% of input number compared to 30 $\pm$ 3%, in a series of 10 experiments). Structural similarity to fMet-Leu-Phe is suggested by the finding that partially purified bacterial peptides inhibit binding of fMet-Leu-Phe-Lys-FITC to human monocytes. The objective this year was to purify and determine the structure of the most active peptide. A single peak of chemotactic activity was obtained by sequential purification by reverse phase HPLC on C-18 and diphenyl columns. The amount of peptide in this peak was below the detection limit of the most sensitive amino acid analysis, indicating that the peptide is effective as an attractant at 10^{-10}M or less. Since no structural data will be forthcoming without a greatly increased amount of starting material, production of bacterial culture fluid has been increased from 1 liter to 75 liters, in preparation for large scale purification.

Although all human neutrophils have receptors for fMet-Leu-Phe, this laboratory found that only 20-40% migrate in vitro at optimal attractant concentrations. fMet-Leu-Phe can be oxidized (and inactivated as an attractant) by myeloperoxidase (MPO) released from stimulated neutrophils. We determined if the chemoattractant efficacy of fMet-Leu-Phe (measured as

the percentage of neutrophils migrating) could be increased by protection from oxidation. Addition of 10^{-4}M sodium ascorbate or mercaptoethanol (2ME) to the attractant well increased efficacy 2-3 fold. Similar results were obtained by addition of agents with oxidizable sulfur, 10^{-5}M methionine or $2x10^{-6}$M albumin. Catalase, which destroys H_2O_2, also increased efficacy; superoxide dismutase, which forms H_2O_2 from superoxide, did not. Two experiments suggested that the antioxidant effect was on the attractant rather than the cells: albumin in the cell compartment did not increase fMet-Leu-Phe responses; antioxidants in either cell or attractant well had no effect on efficacy of fNle-Leu-Phe-Nle-Tyr-Lys, an attractant that also stimulated superoxide release but had no oxidizable sulfur. Thus, the low efficacy of fMet-Leu-Phe is due in part to oxidation by migrating neutrophils. The concentration of albumin in tissue spaces is sufficient to protect attractants from oxidative inactivation in vivo.

Publications:

Leonard, E.J., Skeel, A., and Alteri, E. Human monocyte chemotaxis: functional and flow cytometric analysis. In Macrophage Biology, Reichard, S. and Kojima, M. eds. pp 259-270. Alan Liss, N.Y., 1985.

Leonard, E.J. and Skeel, A. Separation of human basophils into two fractions with different density and histamine content. J. Allergy and Clinical Immunology 76:556-562,1985.

Leonard, E.J., Noer, K. and Skeel, A. Analysis of human monocyte chemoattractant binding by flow cytometry. J. Leukocyte Biology 38:403-413,1985.

Leonard, E.J. and Skeel, A. Disposable microliter immunoabsorbent columns: construction and operation. J. Immunol. Methods 82:341-348,1985.

Rot, A., Henderson, L.E. and Leonard, E.J. Staphylococcus aureus derived chemoattractant activity for human monocytes. J. Leukocyte Biol. In press.

Annual Summary Report, Laboratory of Cell Biology
National Cancer Institute

October 1, 1985 - September 30, 1986

The following are selected highlights of accomplishments for the Laboratory of Cell Biology for fiscal year 1986.

We have purified to homogeneity two tumor specific transplantation antigens (TSTA) from a BALB/c methylcholanthrene-induced tumor, Meth A. Two TSTAs were identified having molecular masses of 82,000 (p82) and 86,000 (p86) by SDS polyacrylamide gel electrophoresis. These antigens were found to retain their specific in vivo tumor rejecting activity. The p86 and p82 TSTAs do not appear to be related since they exhibit different molecular size and charge (pI), antisera to each protein do not show crossreactivity and, finally, each has a unique amino terminal sequence. Using antisera to these antigens, we have found proteins of identical size and charge in normal mouse tissues as well as other transformed cells. Immunofluorescence as well as electron microscopic analysis of the Meth A TSTA, using affinity-purified antisera, demonstrated that both antigens are mainly found in the cytoplasm but are also present at the cell surface; thus the antigens are accessible to the hosts immune system. Biochemical analysis indicated that neither TSTA is a glycoprotein which raises the question of how a cytoplasmic protein becomes associated with the cell surface. The p86 TSTA was found to actually consist of two protein species having M_rs of 86 and 84 kD. A TSTA apparently identical to the Meth A 86 and 84 kD antigens has been isolated from two other syngeneic tumors, one a methylcholanthrene-induced tumor, CI-4 and the other an SV40 induced tumor, mKSA. Each behaves as a TSTA. p82 was also purified from CI-4 and mKSA but the yield was too low for adequate immunogenic and specificity assays.

The amino terminal sequence of the 86- and 84- kD antigens were found to be identical except that each chain contained a portion of a unique sequence. Comparison of the NH_2- terminal and CNBr fragment sequence of p86 show extensive homology to yeast and Drosophila melanogaster heat shock proteins (~80% homology). The following evidence indicates that the p86 TSTA from Meth A, as well as CI-4 and mKSA tumors, are related to or identical to the major 85 kD murine stress protein: 1) antisera to the Meth A TSTA p86 specifically immunoprecipitates the 85 kD stress protein from heat shocked mouse embryo cells having molecular masses and pIs identical to those of the purified TSTA; 2) the 85 kD heat shock protein and its equivalent in other species are phosphoproteins as are the Meth A TSTAs; 3) the murine 85 kD has also been reported to be a protein doublet.

Amino acid sequence analysis of the 86- and 84 kD TSTA from CI-4 and mKSA tumors has been started and the antigens have been found to have NH_2-terminal sequences identical to their respective Meth A counterparts.

In order to define the molecular changes which render the heat shock protein, expressed by the 3-methylcholanthrene-induced tumors, a TSTA, we have undertaken the cloning and sequence analysis of the corresponding nucleic acid. We have isolated a cDNA clone corresponding to one of the isoforms of p86. The amino acid sequence deduced from the coding region of this clone shows homology to the 83 kD and 90 kD heat shock proteins of D. melanogaster and S. cerevisiae, respectively. A segment from this clone was used to screen cDNA

and genomic libraries from the same and other tumors. Isolation and molecular analysis of additional clones is in progress. Comparison of Northern and Southern blots of restriction enzyme digests of normal and tumor cells indicates that the genes coding for 86 kD heat shock protein are not grossly altered in the neoplastic state. This finding gives additional evidence that the molecular changes which confer TSTA activity are most likely single or limited amino acid substitutions. Further sequence analysis hopefully will elucidate the nature of the antigenic modification in different tumors which elicit specific T cell immunity.

Recently, from a newly induced MC tumor, CII-7, two tumor-specific antigens of 82 kD and 86 kD molecular weights have been isolated and purified to chemical homogeneity. We used the procedures of hexylamine agarose chromatography, Sepharose S-300 filtration and hydroxylapatite chromatography followed by a final step of HPLC-DEAE chromatography, starting with cytosolic fractions. Both antigens were purified from the same preparation. In in vivo assays, both were found to be highly immunogenic and specific for CII-7, not crossreacting with all other tumors tested. The NH_2- terminal sequence for CII-7 proved to be identical with that determined for Meth A, as follows: H_2N-PKPINVRUTTMDAELEFAIQPN....

The 86 kD protein was first isolated from the SV40-induced mKSA sarcoma and has more recently been purified from the Meth A and CI-4 sarcomas. In each instance the purified 86 kD antigen was found to be immunogenic and specific for the sarcoma from which it was isolated. In the latest study, 86 kD from CII-7 was found to be similar to all others isolated in chromatographic pattern and profile, pI, MW, and exists in two isoforms of 86 kD and 84 kD M_rs and is specific for CII-7 in in vivo immunogenic assays. NH_2 terminal sequences are now being obtained for the CII-7 sarcoma 86 kD protein to determine its relationship to the Meth A protein which is now known to have sequence homology with several heat shock proteins.

Thus, two separate TSTA have been purified to chemical homogeneity, an 82 kD and 86 kD protein, from several sarcomas. All p82s appear to be closely related or similar, as do all p86s, in chromatographic patterns, mass, charge, amino acid composition and sequence; interestingly, each retains immunogenic specificity for the tumor from which it was isolated.

We have demonstrated, previously, that site-directed mutagenesis can be employed to study structure-function relationship of class I histocompatibility antigens. The third external domain of class I antigens has a highly conserved disulfide bridge. To elucidate the functional significance of this disulfide bridge, we have produced a mutant H-2L^d gene in which the codon for cysteine-203 is changed to a codon for serine. No measurable expression of the H-2L^d antigen was found on the cell surface of transformant L-cells. However, a large quantity of the mutant antigen was found in the cytoplasm. Subcellular fractionation indicated that the transport of the antigen was blocked between the endoplasmic reticulum and the plasma membrane. These findings suggest that the structural integrity of the third external domain is important for intracellular transport of class I antigens. The class I heavy chains are N-glycosylated at three sites, one in each external domain. We have constructed mutant H-2L^d antigens in which codons for the N-linked glycosylation sites were replaced by those of other amino acids. The non-glycosylated antigen was unchanged in its serological specificity and was recognized by alloreactive cytotoxic T cells. Further, the antigen was capable of mediating cytotoxic activity of vesicular stomatitis virus-specific T cells.

Cell surface expression of the non-glycosylated antigen was markedly reduced as compared with the native antigen. These findings suggest that the primary role of carbohydrates of class I antigens is to facilitate the intracellular transport of the nascent proteins to the plasma membrane.

Recombinant class I MHC genes were constructed in which the exons of the H-2D^d and H-D^p genes encoding the three extracellular domains of the molecule were shuffled to produce four of the six possible recombinant permutations. These genes were expressed in mouse L-cells by transfection. The reactivities of the recombinant molecules with a large and well characterized panel of anti-H-2D^d monoclonal antibodies were then investigated.

The results confirmed, and extended, the findings from previous experiments with these antibodies and H-2D^d/H-2L^d recombinant molecules. Interestingly, a large proportion of antibodies previously thought to recognize epitopes within the N-domain were now shown not to bind to a construct containing the N-domain of D^d and the C1 domain of D^p. Analysis of the sequences of the C1 domains of D^d, D^p and L^d allowed the identification of a small number of amino acid residues potentially responsible for the generation of these N/C1 complex epitopes. The large number of such antibodies indicates that the region of the N/C1 domain interactions is immunodominant and the patterns of crossreactivity with other class I molecules exhibited by these antibodies shows that they are recognizing many discrete epitopes.

In the last year, we have initiated studies to understand the recognition of lysozymes by T cells. The use of synthetic peptides has permitted the delineation of the fine antigenic specificity of hen egg-white lysozyme (HEL) specific suppressor- and helper- T cells. We have demonstrated that a synthetic peptide corresponding to the N-terminus of HEL induces antibodies which are marginally crossreactive with HEL whereas the same peptide is comparable with the native HEL molecule in immunogenicity and antigenicity at the T helper cell level. Moreover, HEL-specific T suppressor cells in genetically non-responding mice are induced by HEL and the amino terminal synthetic peptide. This induction depends on the presence of phenylalanine at position 3, whereas helper T cells do not discriminate between variants at position 3. These results emphasize the fine interplay between the major histocompatibility complex and antigen-structures in immunoregulatory T cell interactions. The RadLV immortalized suppressor T cell clone LH8.105 specific for lysozyme has been shown to rearrange and to transcribe the genes encoding the α and β subunits of the T cell receptor heterodimer. In order to prove that these genes are capable of producing a functional T cell receptor, a library has been generated in the Okayama Berg expression vector system and α and β chain clones have been isolated. Their sequences are currently being determined. The possession of full length clones will enable us to investigate the role of these genes in the specificity of the Ts clone and of the soluble antigen specific Ts factor which it produces.

There is now evidence that loop and cruciform structures of DNA may have important genetic functions in terms of protein and drug recognition. The self-complementary structure, d-CGCGAATTCGCG, was chosen by Dickerson et al. for an X-ray crystallographic study because it contains an EcoR1 cleavage site. The structure of the duplex was found to be essentially a classic B-form. There have been several studies of oligonucleotide sequences that appear to form hairpin loops. We became interested in studying the tridecamer d-CGCGAATTACGCG which contains an extra A at position 9 compared to the Dickerson sequence and which could potentially have a hairpin loop conformation.

Our results, by proton NMR spectroscopy, indicate that in solution there is a slow equilibration between loop and duplex forms. The mechanism of interconversion involves complete strand separation and reformation rather than cruciform formation and branch migration. Other similar oligonucleotide sequences which may form hairpin loops have been synthesized for structural studies. We have obtained crystals from one of them, d-CGCGAAATTTACGCG. From the crystallographic analysis of this and other hairpin loop sequences, we hope to be able to elucidate the potential molecular mechanism involved in genetic regulation and mutation.

Our continuing studies have shown that the chemically induced JB/RH and JB/MS melanomas, the ultraviolet light induced K1735 melanoma, and the spontaneous B16 melanoma, produce tumor specific transplantation antigens (TSTA) which not only induce autologous tumor rejection, but share determinants with the heterologous melanoma cell lines as well. Our specificity studies have shown that the distribution of these antigens is restricted to the melanoma cells, and no crossreactivity with any non-melanoma tumor lines has been demonstrable. Interestingly, the spontaneous S91 melanoma, although highly immunogenic, does not share cell surface TSTA with other melanoma lines noted above. In order to identify and characterize the relevant TSTA, we have extracted the melanoma cells with aqueous butanol, which quantitatively extracts the antigens, and provides an excellent initial solubilization and purification step. The melanoma specific TSTA have now been further purified by salt fractionation and gel filtration, and have an apparent molecular weight of 50 to 80 kD. Final purification of the TSTA to homogeneity is in progress using preparative gel electrophoresis and high performance liquid chromatography; at that time a final assessment of the multiplicity of the TSTA can be made, and biochemical characterization will be initiated. These crossreacting TSTA (from the B16, JB/RH, JB/MS and K1735 melanomas) are extremely interesting since those tumors were generated by radically different mechanisms, and the TSTA do not display the typical unique specificity previously described for other chemically and ultraviolet light induced neoplasms. In addition, the S91 melanoma provides a comparative model for the study of TSTA with completely restricted specificity.

It has been shown that B700, a melanoma tumor specific antigen (TSA) can elicit tumor rejection activity; immunization of mice with that antigen provided specific and complete, titratable protection against subsequent subcutaneous challenge with B16, JB/RH, and K1735 melanoma cells. Perhaps more importantly, following the intravenous challenge with B16 cells, immunized animals were also competent to significantly reduce the number of subsequent experimental pulmonary metastases. The mechanisms involved in host resistance to tumor challenge following immunization with intact cells, or with TSTA containing fractions, is currently not well understood. Our laboratory has been attempting to characterize the nature of this response by examining T and B cell activity in immunized hosts, and work is underway to isolate specific, activated T cell clones to be used as probes for studying cell mediated immune response mechanisms. We are also examining the nature of the syngeneic cytotoxic antibodies produced in order to study the humoral immune responses following immunization. These studies should yield information not only on the nature of the immunogenic mechanisms involved in tumor rejection by immunized hosts, but perhaps more significantly, will allow us to examine why these mechanisms are typically ineffective in dealing with tumor outgrowth in naive hosts.

Membrane proteins from transformed melanocytes vary in structure from those of normal melanocytes, and even from other types of transformed cells; it has been found that TSA released from melanoma cells can be detected in the serologic fluids of tumor bearing patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro. We have recently shown that one of these shed antigens (B700) has structural homology to the albumins, and may represent a normally occurring gene abnormally expressed in neoplastic tissues. We have found that the primary structure of the B700 expressed on the cell surface of B16 cells differs after serial transplantation of the tumor, and may represent selective outgrowth of various subpopulations of the melanoma cells. B700 isolated from sequential preparations of melanoma tumors passaged in vivo differed significantly in several regions of their amino terminal sequences, and this variability in antigen expression may be responsible, at least in part, for the diversity of subpopulations of melanoma cells that can be detected immunologically within a single tumor. In addition, the ability of this TSA to function as a TSTA has now been demonstrated. Thus, there are implications that the expression of these tumor specific proteins may have a critical immunologic importance to the survival of the tumor in the host. Another melanoma TSA has been isolated and partially characterized; this antigen (B50) did not display significant sequence homology to any other proteins in the sequence data base, nor was it capable of eliciting TSTA activity. These data reinforce the specificity of the results discussed above regarding the B700 antigen, and eliminate the possibility of nonspecific stimulation of the host's immune system causing the tumor rejection activity noted with that antigen.

Plasminogen activators regulate extracellular proteolysis which is important for many biological processes. Two types of plasminogen activators are known, tissue type (tPA) and urokinase plasminogen activator (uPA). Normal and neoplastic cells possess a specific uPA receptor which may serve to focus the proteolytic activity of uPA at the cell membrane. We have shown, by using synthetic peptides, that the region of human and mouse urokinase responsible for binding to their receptors is located within the first 40 amino acid residues (the growth factor module). This region shares partial amino acid sequence homology to the region in epidermal growth factor responsible for receptor binding. The region of homology includes the complete conservation of a heptapeptide sequence and the spacing of the cysteine residues. In the case of mouse and human uPA, the non-homologous regions have been found to confer receptor binding specificity. Similar sequence homology has been found in various other proteins; this homologous region may define a general receptor/ligand recognition module.

We have initiated studies aimed at elucidating the role of cell surface membrane expression of uPA on tumor invasion and metastasis; this enzyme has been postulated to be critical to the process of tumor cell's ability to penetrate membranes and other proteinaceous barriers during metastasis. Consistent with this theory, we have demonstrated that alteration of uPA activity on the melanoma cell surface had dramatic consequences on the subsequent metastatic behavior of these cells. Preincubation of B16 melanoma cells with uPA specific antibodies which inhibit uPA enzymatic activity significantly reduced the metastatic potential of those cells, while other uPA specific antibodies which stimulate the conversion of enzymatically inactive pro-uPA to active uPA actually enhanced metastatic potential. uPA on the cell

surface is bound to receptors, and the binding of active uPA, either exogenously or endogenously derived, may be critical to the penetration of the metastasizing cells through the endothelial capillary wall. The mechanisms involved in these phenomena have not yet been elucidated and are still under active study.

Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, yet rates of pigmentation are influenced at many levels, including hormonal (melanocyte stimulating hormone, α-MSH) and environmental (ultraviolet light), the melanocyte provides a unique model system for the study of intracellular control mechanisms. It has always been questioned how tyrosinase, which is present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is inhibited, but subsequently activated once delivered *in situ* to the melanosome. Our studies have demonstrated that 1-3,4-dihydroxyphenylalanine (1-DOPA) is a natural activator of the enzyme *in vivo*; we have shown that tyrosinase is extremely specific in its requirement for 1-DOPA as a cofactor, which apparently acts as an allosteric regulator. Evidence has been provided that the synthesis of pigment may be further controlled by an, as yet, uncharacterized enzyme-associated factor(s), and that these may prove to be critical to the overall rate of pigment production in mammals (or to the lack of melanogenesis, such as is evident in pigmentary disorders such as albinism and vitiligo). We have now been able to produce tyrosinase specific monoclonal antibodies which crossreact among mammalian tyrosinases, and are proving to be invaluable for the characterization of post-translational enzyme processing in melanocytes. These probes are being employed by many laboratories for the specific and sensitive identification of murine and human melanocytes *in vivo* and *in vitro* (in both normal and transformed populations). We have examined the metabolic processing of tyrosinase, which involves glycosylation and intracellular transport, as a model for regulatory control of mammalian cells. We have found that tyrosinase is synthesized, glycosylated and transported through the Golgi apparatus to the melanosome much more rapidly than previously thought, and occurs within minutes. This model system has been used to analyze the response mechanisms of melanocytes to α-MSH; it has been found that the response is biphasic, resulting in the activation of preexisting tyrosinase as an immediate response, and the initiation of increased synthesis of the enzyme as a delayed response (taking approximately 48 hr).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03200-17 LCBGY

PERIOD COVERED

October 1, 1985 through September 30, 1986

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)*

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3	1	2

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Major emphasis is placed upon the study of Class I (restricted) and Class II (crossreacting) tumor antigens of the transplantation rejection type (TATA), and of tumor antigens (TA) assayed by in vivo and in vitro techniques and of the immune responses they evoke. As a corollary to this study, the biologic properties in vitro and in vivo of alien histocompatibility (H-2) antigens and of variant antigens in several neoplasms are under study. Solubilization and methods of purification of TATAs are under investigation with the ultimate purpose of defining these membrane and cytosol antigens, after purification, in physicochemical, biologic and molecular terms.

Major Findings:

STUDIES OF PURIFICATION AND OF THE CHEMICAL AND BIOLOGIC PROPERTIES OF UNIQUE TUMOR ANTIGENS (TATA)

Two distinct TATAs have now been purified to chemical homogeneity from Meth A and CI-4, both chemically-induced murine sarcomas. The 82 kD antigen was described several years ago as a 75 kD antigen; recent isolations from several tumors and further studies now reveal the molecular mass of this antigen to be 82 kD. The purified Meth A protein, as determined by two-dimensional gel analysis and NH_2- and COOH-terminal sequencing, retains its unique immunogenicity and specificity by *in vivo* testing in syngeneic mice. 82 kD has been purified from a newly-arisen sarcoma, CII-7, and this is discussed later. The NH_2- and COOH-termini analyses of the Meth A 82 kD protein indicate it to be a pure protein consisting of a single polypeptide chain. Comparison of these sequences, along with several cyanogen bromide digests, indicates this protein to have little sequence homology to other known proteins.

From a newly induced MC tumor, CII-7, two tumor-specific antigens of 82 kD and 86 kD molecular weights have been isolated and purified to chemical homogeneity (with DuBois, FCRF). We used the procedures of hexylamine agarose chromatography, Sepharose S-300 filtration and hydroxylapatite chromatography followed by a final step of HPLC-DEAE chromatography, starting with cytosolic fractions. Both antigens were purified from the same preparation. In *in vivo* assays, both were found to be highly immunogenic and specific for CII-7, not crossreacting with all other tumors tested. The NH_2- terminal sequence for CII-7 proved to be identical with that determined for Meth A, as follows: H_2N-PKPINVRUTTMDAELEFAIQPN....

The 86 kD protein was first isolated from the SV40-induced mKSA sarcoma and has more recently been purified from the Meth A and CI-4 sarcomas. In each instance the purified 86 kD antigen was found to be immunogenic and specific for the sarcoma from which it was isolated. In the latest study, 86 kD from CII-7 was found to be similar to all others isolated in chromatographic pattern and profile, pI, MW and exists in two isoforms of 86 kD and 84 kD weights and is specific for CII-7 in *in vivo* immunogenic assays. NH_2 terminal sequences are now being obtained for the CII-7 sarcoma 86 kD protein to determine its relationship to the Meth A protein which is now known to have sequence homology with several heat shock proteins (with Appella, Robinson, Ullrich).

Thus, two separate TATA have been purified to chemical homogeneity, an 82 kD and 86 kD protein, from several sarcomas. All p82s appear to be closely related or similar, as do all p86s, in chromatographic patterns, mass, charge, amino acid composition and sequence; interestingly, each retains immunogenic specificity for the tumor from which it was isolated. The table below shows results, to date, of the purification and specific immunogenicity of the p82 and p86/84 TATA.

	p82	p86/84
Meth A	+	+
mKSA	+*	+
CII-7	+	+
CI-4	+*	+

*not assayed for immunogenicity or specificity due to low yield of purified material.

STUDIES OF THE PROPERTIES OF MURINE MELANOMA-SPECIFIC TUMOR ANTIGENS

We have studied the antigens with tumor rejection activity of a series of murine melanomas in different strains of mice and with different etiologies: B16(F10) spontaneous, JB/RH and JB/MS induced with a chemical carcinogen, K1735 (UV-induced) and S91 another spontaneous melanoma (with Hearing). All have been shown to have moderate-to-strong tumor immunogenicity. In contrast to the unique TATA of sarcomas, four of these melanomas show common TATA that immunize against each other. S91, however, has a strong unique TATA that shows an absolute restriction for the autologus S91. These TATA are tissue-specific since no immunologic crossreactivity was observed against sarcomas, leukemias and carcinomas. A TATA has been isolated and partially purified from the B16 melanoma; this has a MW of 65 kD, an isoelectric point of 4.5, is a glycoprotein and shares sequence homology with a normal protein, serum albumin. This antigen has tumor rejection activity and behaves as intact cells in immunizing also against other melanomas, except S91. Immunization with the 65 kD glycoprotein also effectively prevents experimental metastases to the lungs.

The shared antigens that have been described on human melanotic tissues and that show a pattern of reactivity, as determined by Mabs and serologic assays, are for the most part glycoproteins and glycolipids containing highly complex saccharide structures. These tend to be "differentiation" antigens found at different stages of normal and malignant melanocytes in tissue culture. As such they appear to be quite different from what we observe among the TATA of murine melanomas.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03229-17 LCBGY

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TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural Analysis of Histocompatibility and Tumor Antigens and T-cell Receptors

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

The major area of our research involves the molecular structure of histocompatibility antigens, T cell suppressor receptors and tumor antigens using a combination of protein and DNA sequencing in conjunction with peptide and nucleotide synthesis. Oligonucleotide directed site mutagenesis and recombinant DNA constructs have been employed to elucidate the role of individual amino acids as well as of the individual domains on the function and expression of class I histocompatibility antigens.

A cDNA library from a rearranged T cell suppressor clone specific for lysozyme has been generated and α and β T cell receptor clones have been isolated. These clones will enable us to investigate the role of these genes in the mechanism of T cell suppression.

A tumor specific transplantation antigen from a methylcholanthrene-induced tumor has been identified as a heat shock protein by partial amino acid and DNA sequence analysis. Further analysis should permit identification of the molecular change in this antigen which elicit cell mediated immunity.

DNA sequences predicted to have aberrant helices with inserted bases, forming hairpin stem and loops, have been crystallized. These structures may serve as a model indicating why certain sequences have a much greater tendency to mutate than others and how proteins recognize particular regions of DNA.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

HISTOCOMPATIBILITY ANTIGENS

The major histocompatibility class I antigens are a highly polymorphic set of integral cell surface glycoproteins involved in many aspects of the immune response. They are composed of a heavy chain which has three external domains (N, C1 and C2, numbering from the amino terminus), a transmembrane region and a cytoplasmic tail and is associated through the C2 domain with β_2-microglobulin, an invariant polypeptide encoded outside the MHC. Previously we demonstrated that site-directed mutagenesis can be employed to study structure-function relationship of class I antigens. The third external domain of class I antigens has a highly conserved disulfide bridge. To elucidate the functional significance of this disulfide bridge, we have produced a mutant H-2L^d gene in which the codon for cysteine-203 is changed to a codon for serine. No measurable expression of the H-2L^d antigen was found on the cell surface of transformant L-cells. However, a large quantity of the mutant antigen was found in the cytoplasm. Subcellular fractionation indicated that the transport of the antigen was blocked between the endoplasmic reticulum and the plasma membrane. These findings suggest that the structural integrity of the third external domain is important for intracellular transport of class I antigens.

Mouse class I heavy chains are N-glycosylated at three sites, one in each external domain. We have constructed mutant H-2L^d antigens in which codons for the N-linked glycosylation sites were replaced by those of other amino acids. The non-glycosylated antigen was unchanged in its serological specificity and was recognized by alloreactive cytotoxic T cells. Further, the antigen was capable of mediating cytotoxic activity of vesicular stomatitis virus-specific T cells. Cell surface expression of the non-glycosylated antigen was markedly reduced as compared with the native antigen. These findings suggest that the primary role of carbohydrates of class I antigens is to facilitate the intracellular transport of the nascent proteins to the plasma membrane.

Recombinant class I MHC genes were constructed in which the exons of the H-2D^d and H-2D^p genes encoding the three extracellular domains of the molecule were shuffled to produce four of the six possible recombinant permutations. These genes were expressed in mouse L-cells by transfection. The reactivities of the recombinant molecules with a large and well characterized panel of anti-H-2D^d monoclonal antibodies were then investigated.

The results confirmed, and extended, the findings from previous experiments with these antibodies and H-2D^d/H-2L^d recombinant molecules. Interestingly, a large proportion of antibodies previously thought to recognize epitopes within the N-domain were now shown not to bind to a construct containing the N-domain of D^d and the C1 domain of D^p. Analysis of the sequences of the C1 domains of D^d, D^p and L^d allowed the identification of a small number of amino acid residues potentially responsible for the generation of these N/C1 complex epitopes. The large number of such antibodies indicates that the region of the N/C1 domain interactions is immunodominant and the patterns of crossreactivity with other class I molecules exhibited by these antibodies shows that they are recognizing many discrete epitopes.

T CELL ANTIGEN RECOGNITION

The immune response to a variety of proteins has been extensively investigated to elucidate the mode of recognition of antigens by T cells. In the last year, we have started studies to understand the recognition of lysozymes by T cells. The use of synthetic peptides has permitted the delineation of the

fine antigenic specificity of hen egg-white lysozyme (HEL) specific suppressor- and helper- T cells. We have demonstrated that a synthetic peptide corresponding to the N-terminus of HEL induces antibodies which are marginally crossreactive with HEL whereas the same peptide is comparable with the native HEL molecule in immunogenicity and antigenicity at the T helper cell level. Moreover, HEL-specific T suppressor cells in genetically non-responding mice are induced by HEL and the amino terminal synthetic peptide. This induction depends on the presence of phenylalanine at position 3, whereas helper T cells do not discriminate between variants at position 3. These results emphasize the fine interplay between the major histocompatibility complex and antigen-structures in immunoregulatory T cell interactions.

The RadLV immortalized suppressor T cell clone LH8.105 specific for lysozyme has been shown to rearrange and to transcribe the genes encoding the α and β subunits of the T cell receptor heterodimer. In order to prove that these genes are capable of producing a functional T cell receptor, a library has been generated in the Okayama Berg expression vector system and α and β chain clones have been isolated. Their sequences are currently being determined. The possession of full length clones will enable us to investigate the role of these genes in the specificity of the Ts clone and of the soluble antigen specific Ts factor which it produces.

TUMOR SPECIFIC TRANSPLANTATION ANTIGENS

Tumors induced by chemical carcinogens express tumor specific transplantation antigens (TSTAs). A characteristic of these antigens is their polymorphism; immunization of syngeneic hosts with these antigens will elicit cell-mediated immunity, inhibiting the growth only of the tumor from which the antigen was obtained and not against other syngeneic tumors. We have identified and purified to homogeneity two TSTAs from a BALB/c methylcholanthrene-induced tumor, Meth A. The two TSTAs have molecular masses of 82,000 (p82) and 86,000 (p86) by SDS polyacrylamide gel electrophoresis. These antigens were found to retain in vivo tumor rejecting activity. The p86 and p82 TSTAs appear not to be related since they exhibit different molecular size and charge (pI), antisera to each protein does not show crossreactivity and, finally, each has a unique amino terminal sequence. Using antisera to these antigens, we have found proteins of identical size and charge in normal mouse tissues as well as other transformed cells. Thus, these antigens appear to be expressed in differentiated as well as transformed cells. Immunofluorescence as well as electron microscopic analysis of the Meth A TSTAs demonstrated that both antigens are mainly found in the cytoplasm but are also present at the cell surface; thus the antigens are accessible to the hosts immune system. Biochemical analysis indicated that neither TSTA is a glycoprotein. This raises the question of how a cytoplasmic protein becomes associated with the cell surface. The p86 TSTA was found to actually consist of two proteins species having M_rs of 86 and 84 kDa. A TSTA apparently identical to the Meth A 86 and 84 kDa antigens can be isolated from two other syngeneic tumors, one, another methylcholanthrene induced tumor, CI-4, and the other an SV40 induced tumor, mKSA and each behaves as a TSTA.

The amino terminal sequence of the Meth A 86- and 84- kDa antigens were found to be identical except that each chain contained a portion of unique sequence. The NH_2- terminal and CNBr fragment sequences of p86 show extensive homology to yeast and Drosophila melanogaster heat shock proteins (~80% homology). The following evidence indicates that the p86 TSTA from Meth A, as well as CI-4 and mKSA tumors, are related to or identical to the major 85 kDa murine stress protein: 1) antisera to the Meth A TSTA p86 specifically

immunoprecipitates the 85 kDa stress protein from heat shocked mouse embryo cells having molecular masses and pIs identical to those of the purified TSTA; 2) the 85 kDa heat shock protein and its equivalent in other species are phosphoproteins as are the Meth A TSTAs. The murine 85 kDa has also been reported to be protein doublet. Until now this was believed to be due to post-translational modification; however, our results unequivocally demonstrate for the first time that the two proteins represent two protein isoforms. Amino acid sequence analysis of the 86- and 84 kDa TSTA from CI-4 and mKSA tumors has been started and the antigens have been found to have NH_2-terminal sequences identical to their respective Meth A counterparts.

Similar analysis of the p82 antigen has been hampered by the much lower amounts of this antigen present in the CI-4 and mKSA tumors. However, an interesting observation is the inability to detect the p82 protein in secondary mouse embryo cells using the p82 antisera, whereas we have found this protein expressed in differentiated mouse cells. Biochemical analysis of p82 indicated that it is not a phosphoprotein or a heat shock protein. The properties of p82 suggest that it may be a differentiation antigen.

In order to define the molecular changes which render the heat shock protein, expressed by the 3-methylcholanthrene-induced tumors, a TSTA, we have undertaken the cloning and sequence analysis of the corresponding nucleic acid. We have isolated a cDNA clone corresponding to one of the isoforms of p86. The amino acid sequence deduced from the coding region of this clone shows homology to the 83 kDa and 90 kDa heat shock proteins of D. melanogaster and S. cerevisiae, respectively. A segment from this clone was used to screen cDNA and genomic libraries from the same and other tumors. Isolation and molecular analysis of additional clones is in progress.

Comparison of Northern and Southern blots of restriction enzyme digests between normal and tumor cells indicates that the genes coding for 86 kDa heat shock protein are not grossly altered in the neoplastic state. This finding gives additional evidence that the molecular changes which confer TSTA activity are most likely single or limited amino acid substitutions. Further sequence analysis hopefully will elucidate the nature of the antigenic modification in different tumors which elicit T cell immunity.

PROTEIN AND NUCLEIC ACID CHEMISTRY

Myosin from Acanthamoeba castellanii has been studied extensively and it has a structure similar to that of other muscle and non-muscle myosins. It has previously been shown that the active site of myosin can be photoaffinity-labeled by the substrate [5,6-^{3}H]UTP. We have now determined the amino acid sequence of the single-labeled peptide that is produced by extensive tryptic digestion. The following amino acid sequence was obtained:

Thr-Gln-Asn-Thr-Me_2Lys-Lys (where Me_2Lys represents dimethylysine)

This sequence has homology to an analogous region of rabbit skeletal muscle and nematode myosin heavy chain, which suggests structural or functional importance for this region.

Plasminogen activators regulate extracellular proteolysis which is important for many biological processes including metastasis. Two types of plasminogen activators are known, tissue type (tPA) and urokinase (uPA). Normal and neoplastic cells possess a specific uPA receptor which may serve to focus the proteolytic activity of uPA at the cell membrane. We have shown, by using synthetic peptides, that the region of human and mouse urokinase responsible for binding to their receptors is located within the first 40 amino acid residues (the growth factor module). This region shares partial amino acid sequence

homology to the region in epidermal growth factor responsible for receptor binding. The region of homology includes the complete conservation of a heptapeptide sequence and the spacing of the cysteine residues. In the case of mouse and human uPA, the non-homologous regions have been found to confer receptor binding specificity. Similar sequence homology has been found in various other proteins; this homologous region may define a general receptor/ligand recognition module.

During the past few years, there has been growing evidence that loop and cruciform structures of DNA may have important genetic functions in terms of protein and drug recognition. The self-complementary structure, d-CGCGAATTCGCG, was chosen by Dickerson et al. for an X-ray crystallographic study because it contains an EcoR1 cleavage site. The structure of the duplex was found to be essentially a classic B-form. There have been several studies of oligonucleotide sequences that appear to form hairpin loops. We became interested in studying the tridecamer d-CGCGAATTACGCG which contains an extra A at position 9 compared to the Dickerson sequence and which could potentially have a hairpin loop conformation. Our results, by proton NMR spectroscopy, indicate that in solution there is a slow equilibration between loop and duplex forms. The mechanism of interconversion involves complete strand separation and reformation rather than cruciform formation and branch migration.

Other similar oligonucleotide sequences which may form hairpin loops have been synthesized for structural studies. We have obtained crystals from one of them, d-CGCGAAATTTACGCG. From the crystallographic analysis of this and other hairpin loop sequences, we hope to be able to elucidate potential molecular mechanism envolved in genetic regulation and mutation.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09100-3 LCBGY

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October 1, 1985 to September 30, 1986

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

Immunogenicity of Melanoma

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LAB/BRANCH

Laboratory of Cell Biology

SECTION

Chemistry

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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.)*

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. The results indicate that various murine melanomas (of spontaneous, ultraviolet light induced, and chemically induced origin) share common cell surface antigens which are capable of eliciting tumor rejection; these antigens have a specificity restricted to melanoma cells. One melanoma tumor specific antigen has been purified to homogeneity and partially characterized; it is a glycoprotein of 65 kilodaltons, which has biochemical and immunological homology to serum albumin; it has been shown to be effective in immunizing mice against subsequent challenge with melanoma in transplantation assays and demonstrates the potential immunological significance of tumor specific antigen expression on the progress of tumor growth. We have found that alteration of surface urokinase activity significantly affects the metastatic potential of melanoma cells, suggesting that this protease plays an important role in the metastatic sequence. We have produced and utilized monoclonal antibodies specific for the melanocyte specific enzyme, tyrosinase, to examine cellular control mechanisms functional in the response of melanocytes to varying environmental stimuli which affect pigmentation, such as melanocyte stimulating hormone.

PHS 6040 (Rev. 1/84)

GPO 904-917

Major Findings:

IMMUNOGENICITY OF MALIGNANT MELANOMA

Our studies (with Gersten, Law et al.) have shown that the chemically induced JB/RH and JB/MS melanomas, the ultraviolet light induced K1735 melanoma, and the spontaneous B16 melanoma, produce tumor specific transplantation antigens (TSTA) which not only induce autologous tumor rejection, but share determinants with the heterologous melanoma cell lines. Our specificity studies have shown that the distribution of these antigens is restricted to melanoma cells, and no cross-reactivity with non-melanoma tumor lines has been demonstrable (except a single neuroblastoma cell line, which has an identical embryologic derivation). Interestingly, the spontaneous S91 melanoma, although highly immunogenic, does not share cell surface TSTA with the other melanoma lines. In order to identify and characterize the relevant TSTA, we have extracted melanoma cells with aqueous butanol, which quantitatively extracts the antigens, and provides an excellent initial solubilization and purification step. The melanoma specific TSTA have now been further purified by salt fractionation and gel filtration, and have a molecular weight of 50 to 80 kilodaltons. Final purification of the TSTA to homogeneity is in progress using preparative gel electrophoresis and high performance liquid chromatography; after final purification, an assessment of the multiplicity of melanoma TSTA will be made, and biochemical characterization will be initiated. These cross-reacting TSTA (from the B16, JB/RH, JB/MS and K1735 melanomas) are extremely interesting since those tumors were generated by radically different mechanisms, yet the TSTA do not display the typical unique specificity previously described for other chemically and ultraviolet light induced neoplasms. In addition, the S91 melanoma provides a comparative model for the study of TSTA with restricted specificity.

We have shown that B700, a melanoma tumor specific antigen (TSA), can elicit tumor rejection activity; immunization of mice with that antigen provided specific and complete, titratable protection against subsequent subcutaneous challenge with B16, JB/RH, and K1735 melanoma cells. In addition, following intravenous challenge with B16 cells, immunized animals were also competent to significantly reduce the number of subsequent experimental pulmonary metastases. The mechanisms involved in host resistance to tumor challenge following immunization with intact cells, or with TSTA containing fractions, is currently not well understood. Our laboratory has been attempting to characterize the nature of this response by examining T and B cell activity in immunized hosts, and work is underway to isolate specific, activated T cell clones to be used as probes for studying cell mediated immune response mechanisms (with Kameyama and Bookman). We are also examining the nature of cytotoxic antibodies produced in order to study humoral immune responses following immunization (with Law). These studies should yield information not only on the nature of the immunogenic mechanisms involved in tumor rejection by immunized hosts, but perhaps more significantly, will allow us to examine why these mechanisms are typically ineffective in dealing with tumor outgrowth in naive hosts.

TUMOR SPECIFIC ANTIGENS PRODUCED BY MALIGNANT MELANOMA

Membrane proteins from transformed melanocytes vary in structure from those of normal melanocytes, and from other types of transformed cells; it has been found that TSA are released from viable melanoma cells and can be detected in the serologic fluids of tumor bearing patients and mice, and that relatively large quantities of these proteins are shed. We have recently shown that one of

these shed TSA (B700) has structural homology to the albumins, and may represent a normally occurring gene abnormally expressed in neoplastic tissues (with Marchalonis, Schwabe and Gersten). We have found that the primary structure of B700 expressed on the surface of B16 cells differs after serial transplantion of the tumor, and may represent selective outgrowth of various subpopulations of the melanoma cells. B700 isolated from sequential preparations of melanoma tumors passaged in vivo differed significantly in several regions of their amino terminal sequences, and this variability in antigen expression may be responsible, at least in part, for the diversity of subpopulations of melanoma cells that can be detected immunologically within a single tumor. In addition, the ability of this TSA to function as a TSTA has now been demonstrated (with Gersten and Law). Thus, there are implications that the expression of these tumor specific proteins may have a critical immunologic importance to the survival of the tumor in the host. Another melanoma TSA has been isolated and partially characterized; this antigen (B50) did not display significant sequence homology to any other proteins in the sequence data base, nor was it capable of eliciting TSTA activity; this evidence reinforces the specificity of the results regarding the B700 antigen, and eliminates the possibility of nonspecific stimulation of the host's immune system causing the tumor rejection activity noted with that antigen.

We are also examining the expression and modulation of a B16 TSA defined by monoclonal antibody 3C6 (with Leong). We have found that this TSA was expressed on the JB/RH melanoma, and on 6 sublines of the B16 melanoma, but was not detectable on 3 other murine melanomas, 11 murine nonmelanoma tumors, 9 syngeneic normal murine tissues, or 13 human melanomas examined. The TSA synthesis and expression was maximized as the cells reached confluence, but decreased thereafter; there was no correlation with pigment formation, tyrosinase activity, or H2 surface expression. Histochemical analysis at the electron microscopic level demonstrated that once the antibodies were bound to the surface TSA, they were rapidly internalized. In light of the correlation of the expression of this TSA with the cell cycle, the specificity and the rapid internalization of the antibody, this TSA should prove to be an excellent model for the study of the expression of tumor antigens, their regulation by environmental conditions, and the effective immunomodulation of tumor growth by immunocytotoxic agents.

METASTATIC EVENTS IN DISSEMINATION OF MALIGNANT MELANOMA

We have initiated studies aimed at elucidating the role of cell surface membrane expression of urokinase plasminogen activator (uPA) on tumor invasion and metastasis; this enzyme has been postulated to be critical to the process of the tumor cell's ability to penetrate membranes and other proteinaceous barriers during metastasis. Consistent with this theory, we (with Appella, Blasi and Law) have demonstrated that alteration of uPA activity on the melanoma cell surface had dramatic consequences on the subsequent metastatic behavior of these cells. Preincubation of B16 cells with uPA specific antibodies which inhibit uPA enzymatic activity significantly reduced the metastatic potential of those cells, while other uPA specific antibodies which stimulate the conversion of enzymatically inactive pro-uPA to active uPA actually enhanced metastatic potential. uPA on the cell surface is bound to specific receptors (with Appella, Blasi and Stoppelli), and the binding of active uPA, either exogenously or endogenously derived, may be critical to the ability of metastasizing cells to penetrate the endothelial capillary wall to enter and exit the circulatory

system. Although the mechanisms involved in these phenomena have not yet been elucidated and are still under active study, these results have shown that uPA plays an important role in process of metastasis.

REGULATION OF MELANOGENESIS

The melanocyte provides a unique model system for the study of intracellular control mechanisms since only the enzyme tyrosinase is essential for melanin biosynthesis, yet rates of pigmentation are influenced at many levels, including hormonal (melanocyte stimulating hormone, α-MSH) and environmental (ultraviolet light). It has always been questioned how tyrosinase, which is present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is enzymatically inhibited, but subsequently activated once delivered in situ to the melanosome. Our studies have demonstrated that 1-3,4-dihydroxyphenylalanine (1-DOPA) is a natural activator of the enzyme in vivo; we have shown that tyrosinase is extremely specific in its requirement for 1-DOPA as a cofactor, which acts as an allosteric regulator. We have provided evidence that the synthesis of pigment may be further controlled by enzyme-associated factor(s), and that these may prove to be critical to the overall rate of pigment production in mammals (or to the lack of melanogenesis, such as is evident in pigmentary disorders such as albinism and vitiligo). We have been able to produce tyrosinase specific monoclonal antibodies which cross-react among mammalian tyrosinases, and are proving to be invaluable for the characterization of post-translational enzyme processing in melanocytes. These probes are being employed by many laboratories for the specific and sensitive identification of murine and human melanocytes in vivo and in vitro (in both normal and transformed populations). We have examined the metabolic processing of tyrosinase, which involves glycosylation and intracellular transport, as a model for regulatory control of mammalian cells (with Jimenez and Tomita). We have found that tyrosinase is synthesized, glycosylated and transported through the Golgi apparatus to the melanosome much more rapidly than previously thought, and occurs within minutes. We have used this model system to analyze the response mechanisms of melanocytes to α-MSH, and have found that the response is biphasic, resulting in the activation of preexisting tyrosinase as an immediate response, and the initiation of increased synthesis of the enzyme as a delayed response (taking approximately 48 hr). Treatment of human melanoma cells with Y-interferon showed that although this had no significant effect on tyrosinase activity or synthesis alone, it had a dramatic synergistic positive effect on melanogenesis when employed in conjunction with α-MSH. Further study of this system with probes now becoming available as the mammalian tyrosinase gene is cloned will enable further clarification of some of the mechanisms involved in the responses to environmental and hormonal stimuli by mammalian melanocytes.

Publications:

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SUMMARY REPORT

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY

October 1, 1985 to September 30, 1986

The Laboratory of Tumor Immunology and Biology carries out a range of laboratory investigations in tumor immunology, tumor cell biology, and molecular biology. Areas of particular emphasis include: (a) biochemical mechanisms of oncogene expression and cell growth; (b) the identification and characterization of cellular genes associated with the development of murine and human mammary neoplasia; and (c) the development and utilization of monoclonal antibodies directed against tumor associated antigens, with emphasis being placed on the study of human mammary and colon carcinomas.

The Laboratory of Tumor Immunology and Biology was established in 1982. It is composed of six Sections: The Cellular and Molecular Physiology Section (Dr. Herbert Cooper, Chief); The Biochemistry of Oncogenes Section (Dr. Robert Bassin, Chief); The Oncogenetics Section (Dr. Robert Callahan, Chief); The Experimental Oncology Section (Dr. Jeffrey Schlom, Chief); The Cellular Biochemistry Section (Dr. Cho-Chung, Chief); The Cell Cycle Regulation Section (Dr. William Kidwell, Chief).

CELLULAR AND MOLECULAR PHYSIOLOGY SECTION (Dr. Herbert Cooper, Chief)

The overall goal of projects currently in progress in this Section is the elucidation of biochemical events involved in regulation of cell growth whose derangement as a consequence of oncogene expression may cause or contribute to neoplastic cell transformation. Three biochemical areas have been emphasized:

1. Alterations in the synthesis of specific cellular proteins as a consequence of expression of retroviral oncogenes in murine (NIH/3T3) cells.

2. Derangements in synthesis and organization of cytoskeletal proteins in human neoplasms and their mode of contribution to the neoplastic state.

3. Phosphorylation events related to the response of human leukemic cells to tumor-promoting phorbol esters.

In Project 1, studies have continued on the role of tropomyosin suppression in neoplastic transformation. To ascertain which, if any, components of the transformed phenotype are directly caused by suppression of tropomyosin synthesis, specific inhibition of translation of tropomyosin mRNA in intact cells was attempted by constructing an anti-sense producing vector and introducing it into NIH/3T3 cells. Successful transfectants were selected by resistance to the antibiotic, G418, and these have now been cloned. By translation of mRNA in cell-free systems and by northern blot analysis, it was established that tropomyosin suppression results from reduced levels of specific tropomyosin mRNAs. To improve the specificity of molecular probes for mouse cell tropomyosin mRNA and DNA, and to obtain more effective anti-sense sequences, a cDNA library derived from NIH/3T3 mRNA was produced in the lambda-gtll expression vector. This library was screened with the TM4 cDNA probe and with anti-tropomyosin

antiserum to select specific cDNA clones for the various species of tropomyosin. Thirty TM4-positive plaques were identified and purified.

In Project 2 the synthesis and content of tropomyosins and other cytoskeletal proteins in various human tumor cell lines and tissues will be investigated in order to determine whether synthesis of these proteins is suppressed in human neoplasms as it is in NIH/3T3 cells transformed by oncogenic viruses. Tropomyosins synthesized by various tissue types will be identified by 2-dimensional polyacrylamide gel electrophoresis on the basis of molecular weight, isoelectric point, cytoskeletal localization, absence of tryptophan and immunological reactivity. Expression of tropomyosins in tumor cells of these types will then be evaluated. Synthesis of other cytoskeletal proteins will be evaluated in the same preparations. The following systems will be studied: lymphoid cells, myeloid cells, colon carcinoma, bladder carcinoma, and mammary carcinonma.

In Project 3 studies have continued on the unique phosphoprotein, pp17, which undergoes rapid phosphorylation in HL60 promyelocytic leukemia cells in response to treatment with phorbol ester (TPA). By tryptic peptide analysis of specific proteins recovered from 2-dimensional electrophoretic gels, the nonphosphorylated precursor (p17) of pp17 has been identified. A novel and inexpensive method for measurement of radioactivity in single proteins directly on 2-dimensional gels was devised in this laboratory by R. Braverman. Using this technique, the cellular content of p17 and the rate and extent of its phosphorylation to pp17 after TPA treatment was quantitated. p17 was found to be one of the major cytosolic proteins in HL60 cells (0.5% of total cytosolic protein). About 50% of preexisting p17 is phosphorylated within 15 minutes of addition of TPA to HL60. These findings indicate that phosphorylation of p17 is one of the most rapid, and quantitatively significant biochemical responses to TPA treatment. Since this occurs in the context of abrupt cessation of cell growth and onset of monocytoid differentiation, the possible role of p17 and pp17 in these processes is being explored. The only other phenomenon known to occur in this time frame in this system is the rapid and transitory activation of the c-fos cellular oncogene. The possibility of a functional interrelationship between these two phenomena is being explored. The possible role of p17 and its phosphorylation to pp17 in cell growth regulation in other cell types is also being explored. Preliminary results indicate increased phosphorylation of p17 in lymphoid cells which exhibit reduced cell growth rate in response to serum deprivation.

BIOCHEMISTRY OF ONCOGENES SECTION (Dr. Robert Bassin, Chief)

The Biochemistry of Oncogenes Section studies the mechanisms of cell transformation and tumor formation by retroviral oncogenes. Two general approaches are employed: analysis of flat revertant cells resistant to transformation by certain groups of oncogenes; and biochemical studies, in which the role of cell membrane components in growth and transformation are examined.

During the past year, studies on revertant cell lines resistant to transformation by the retroviral oncogene ras have been extended. Electron probe studies indicated that ras-transformed NIH/3T3 cells treated with either ouabain or with medium containing potassium exhibited a greatly increased rate of potassium loss than did nontransformed controls. Further, nontransformed revertant cells showed low levels of potassium loss under the same conditions. Analysis of revertant cells indicated that the acquisition at least 2 different patterns of potassium

transport, each manifested by a slower loss of potassium following ouabain treatment, could be associated with the revertant phenotype. One pattern is characteristic of cell mutants containing alterations in the enzyme sodium, potassium ATPase, while the other pattern is similar or identical to that associated with a mouse gene recently identified and cloned be Levenson et al. following transfection of normal mouse cell high molecular weight DNA into human cells. The possibility that alterations in monovalent cation transport is primarily responsible for the ras-induced transformed phenotype is under study.

We have developed additional techniques, including chromium release and crystal violet uptake, to study the effects of ouabain on various normal and transformed cells. The human cell line HOS also exhibits a dramatically increased sensitivity to ouabain following ras-induced transformation, indicating that the mechanism of transformation in these cells also involves ion transport. In the case of human cells, however, sensitivity is dependent upon an alkaline pH, a feature which has not been observed in studies on mouse cells to date. Initial experiments indicate that some human tumor cell lines are more susceptible to the toxic effects of ouabain than other such lines, but the significance of this finding has yet to be brought to light.

In addition to the genetic analysis described above, we are also studying the mechanism of transformation by the identification of important cellular control mechanisms which affect growth and differentiation and by the definition of the transformation process by its effects on these critical control mechanisms. In this section, previous studies have identified protein kinase C (C-kinase) as such a control mechanism. C-kinase appears to be active when bound to the plasma membrane of cells and relatively inactive when present in cytosolic fractions. Membrane binding is enhanced by a variety of agents, including the tumor promoter TPA. We have now developed a reconstitution system using partially purified rat brain C-kinase. Using this system, we show that chelator-stable binding of a TPA-C-kinase complex to the plasma membrane requires calcium and phospholipids and is regulated by calcium levels in intact cells. Addition of the growth factor interleukin-2 also causes a rapid but transient redistribution of C-kinase from cell cytoplasm to plasma membrane and increases the metabolism of phosphoinositol. We have developed an in vitro assay to study the hydrolysis of labelled PI and PIP_2, and results to date indicate that PIP_2 phosphodiesterase activity is enhanced in ras-transformed NIH/3T3 cells. The role of the ras gene product, p21, in the modulation of PIP_2 phosphodiesterase is under investigation.

Two additional projects ongoing in the section are listed below:

Role of Retinoids and Hormones in Mediating Cell Growth and Differentiation

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 cyclic AMP-dependent protein kinase (cAMP-PK) activities. PCC4(RA)-1 and Nulli (RA)-1 are mutant EC cell lines which fail to differentiate in response to retinoic acid. The former, but not the latter, lacks the cellular retinoic acid-binding protein (cRABP). Treatment of Nulli (RA)-1 cells (which possess cRABP) with RA provokes an increase in cAMP-PK activities and cAMP binding, whereas RA treatment of PCC4 (RA)-1 cells (which lack cRABP) does not cause an increase in cAMP-PK activity. These results suggest that cRABP may be required to mediate the RA-induced increase in cAMP-PK activities. Other studies have demonstrated

that human psoriatic fibroblasts have little, if any, R_{II} regulatory subunits when compared to R_{II} levels found with normal human fibroblasts. DEAE cellulose fractionation of extracts prepared from psoriatic fibroblasts also shows decreased type I cAMP-PK activity and the complete absence of type II kinase activity. The amount of the R_I regulatory subunit present in red blood cells obtained from psoriatic patients is significantly decreased compared to R_I levels in red cell membranes from normal subjects. These changes in R_I correlate with the severity of the disease, and indicate that determination of R_I levels in red blood cells may be used as an index to establish the presence and severity of psoriasis. Human EC (Tera 2) cells in culture offer an in vitro system to identify possible hormones and growth factors which might play a role in regulating embryonic growth and differentiation. Results indicate that Tera 2 cells have insulin-like growth factor I (IGF-I) receptors present on the cell surface. Exposure of Tera 2 cells to RA leads to differentiation to a neuronal-like cell type which exhibits enhanced calcitonin stimulation, and somatostatin inhibition, of adenylate cyclase activity. These findings suggest a possible role for IGF-I, calcitonin, and somatostatin during embryonic development.

Identification of Cellular Targets of Oncogene Products

Studies have shown that treatment of intact cells with tumor promoters (TPA), as well as with certain growth factors and hormones, causes a rapid redistribution or stabilization (activation) of protein kinase C to the particulate fraction. Results indicate that part of the PK-C stabilized to the membrane fraction with exposure to tumor promoters may be recovered associated with nuclear-cytoskeletal components. Following treatment of NIH/3T3 cells with TPA for 20 min, PK-C activity is found tightly associated with the highly purified nuclear fraction. Immunohistochemical localization studies further support the observation that TPA treatment mediates association of PK-C to the nucleus and cytoskeleton of NIH/3T3 cells. This TPA-induced redistribution of PK-C to the nuclear fraction is not observed with HL-60 cells. The different subcellular distributions of PK-C induced by TPA with different cell types may help to explain, in part, the different responses noted with various cell types with exposure to TPA. The same type of TPA-induced association of PK-C with membranes can be obtained in a reconstitution system with purified PK-C and isolated plasma membranes in the presence of TPA, Ca^{2+}, and phosphatidylserine. This TPA mediated binding is dependent upon Ca^{2+}, is time and temperature dependent, and reaches saturation at ~ 24 ng PK-C/mg PYS cell membrane protein. NIH/3T3 cells transformed with Kirsten (K_1) murine sarcoma virus have elevated membrane-associated PK-C activity and increased phosphatidylinositol (PI) metabolism relative to control 3T3 cells. In vitro studies with membranes isolated from NIH/3T3 and K_1-3T3 cells indicate PIP_2-phosphodiesterase activity is increased with K_1-SV transformation of NIH 3T3 cells. The PIP_2-PDE enzyme from K_1-3T3 cells also is more responsive to activation by GTP analogues. These results further support the suggestion for the involvement of PK-C activation (membrane association) in regulating cell proliferation, and in tumor promotion and malignant transformation, and also suggest possible modulation of this membrane signal transmission system by ras p21 protein.

ONCOGENETICS SECTION (Dr. Robert Callahan, Chief)

The Oncogenetics Section conducts research to identify and molecularly clone tumor specific and viral genetic elements from human and other mammalian species. The characterization of these recombinant clones includes a description of their organization in cellular DNA, their chromosomal location and their biological

activity in normal and neoplastic tissue. The ultimate aim is to develop an understanding of the genetic changes that occur and their consequences in the etiology of human neoplasia. During the past three years, the Oncogenetics Section has focused its efforts in three areas: (1) characterization of the genetically transmitted (endogenous) mouse mammary tumor virus (MMTV) genomes in inbred and feral strains of mice, and their potential role in tumorigenesis; (2) identification of the cellular genes at risk in mammary tumors induced by infectious MMTV; and (3) characterization of MMTV related sequences in human cellular DNA. We have also begun to directly examine genetic alternations associated human breast cancer.

Mammary Tumorigenesis in Inbred and Feral Mice

BALB/c mice have previously been found to express a unique 1.7 kbp species of MMTV RNA in the lactating mammary gland. In previous studies we have established the chromosomal locations of the endogenous MMTV genomes (designated MTV-6, MTV-8, and MTV-9). Using the feral Czech II mouse strain which lacks endogenous MMTV genomes we have genetically derived sublines which contain only MTV-6, MTV-8, or MTV-9. We have found that only the MTV-6 subline expresses the 1.7 kb MMTV RNA in lactating mammary tissue. RNA:RNA *in situ* hybridization of sections from lactating MTG mouse mammary gland show that this viral genome is expressed in discrete regions of the gland.

We have previously identified a new common integration region (designated int-3) for MMTV in MMTV (Czech II) induced mammary tumors. Recombinant clones of this region of the cellular genome have been obtained. Our recent studies have produced the following observations: (1) 6 out of 32 tumors contain a integrated viral genome at *int*-3, (2) in each case the transcriptional orientation of the viral genome is the same direction, (3) 5' to the integrated viral genome, cellular sequences are transcribed into a 2.4 kb species of RNA, (4) the *int*-3 RNA is found only in tumors with an integrated MMTV genome at this locus and is not found in normal lac mammary tissue, (5) *int*-3 is located 11cM from the H-2 locus on chromosome 17, with the gene order centromere-T-H-2-int-3.

The Identification and Characterization of Human Genes Associated with Neoplasia

We have also previously described a novel family of human endogenous retroviral genomes. The nucleotide sequence of the prototype (HLM-2) has been determined. The results confirm our earlier conclusion based on blot hybridization studies that this class of viral genomes is a mosaic of sequences related to different clases of infectious retroviruses. This novel class of endogenous retroviruses is unrelated to mammalian type C retroviruses. The HLM-2 and the related HLM-25 proviral genomes have been shown to be located on respectively human chromosomes 1 and 5. Since the chimpanzee cellular genome also contains these two proviral genomes the viral integration events must have occurred prior to the divergence of these species.

We have begun to determine the genetic alterations associated with breast carcinomas. This study has shown: (1) significant correlation ($p<.001$) between rare alleles of c-H-*ras*-1 and breast cancer patients, (2) the frequent loss of a c-H-*ras*-1 allele correlates with histopathological grade III tumors ($p<.02$), loss of estrogen and/or progesterone receptors ($p<.01$), patients which later developed a distal metastasis ($p<.05$), and (3) amplification or rearrangement of c-*myc* correlates ($p<.02$) with patients over 51 years of age.

Additionally, studies concerning the cDNA cloning of N-acetyl-glycosamine β1-4 galactosyltransferase have been performed in the Section. A specific glycosyltransferase is required for the synthesis of each of the different disaccharide linkages that are known to be present in the oligosaccharide moieties of glycoproteins and glycolipids, which are referred to as glycoconjugates. Galactosyltransferases, a subgroup of the transferases, constitute a family of enzymes which transfer galactose from UDP-gal to the non-reducing residues of oligosaccharides of various glycoconjugates as well as to monosaccharides. We have initiated the molecular cloning approach to understand the modulation of the galactoslytransferase activity, essential for generating specific cell-surface antigenic determinants. In our previous studies on the gene structural analyses of alpha-lactalbumin, a modifier or a specifier protein of galactosyltransferase, we showed that the domain of alpha-lactalbumin that interacts with galactosyltransferase is coded entirely by a separate exon. In the present studies we have cloned and sequenced a cDNA of bovine N-acetyl glucosamine β1-4galactosyltransferase and addressed the question of whether the family of galactosyltransferases have common structural and sequence features despite their varying specificities. Several sequence-related cDNA clones were isolated. The cDNA insert of one set of clones encodes a protein sequence that includes the amino acid sequences of five peptides of bovine galactosyltransferase. The in vitro-translated products coded by human mRNA hybrid-selected with these sets of clones were immunologically reactive with monoclonal antibodies that recognized human but not the bovine 1-4galactosyltransferase epitopes. The sequence analyses of the cDNA clones from several sets show that the regions of the mRNA coding for either carboxylterminal or other domains of the protein are identical with other cDNA clones which may code for proteins that are structurally and may be functionally related to other galactosyltransferases.

EXPERIMENTAL ONCOLOGY SECTION (Dr. Jeffrey Schlom, Chief)

The major research goals of the Experimental Oncology Section (EOS) include the study of the cell biology and tumor immunology of human carcinomas with emphasis being placed on the study of human breast and colon cancer. It is anticipated that the studies outlined here involving the identification and/or characterization of tumor associated antigens, growth and tumor factors and their receptors, oncogene products and epithelial differentiation antigens within carcinoma cells may lead to: (i) a better understanding of the biologically relevant phenotypes that exist among carcinoma cell populations, (ii) a clearer definition of the multistep processes involved in carcinoma pathogenesis, and (iii) the development of reagents and assays that may eventually prove useful in several areas of the management of specific human carcinomas.

The studies being conducted in the EOS have been divided into several projects as summarized below.

Monoclonal Antibodies Define Carcinoma Associated and 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 human carcinomas. These include: detection of occult tumor cells; further defining the degree of differentiation of "normal", dysplastic, and carcinoma

cell populations; serum antigens assays; and radiolocalization of primary and metastatic carcinoma lesions in situ (and potentially therapy) using radiolabeled monoclonal immunoglobins and fragments.

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 for the detection and differentiation of carcinomas from benign cell types and other cancerous lesions.

Oncogene Expression in Human Carcinomas

Monoclonal antibodies (MAbs) of predefined specificity have been generated by utilizing a synthetic peptide reflecting amino acid positions 10-17 of the Hu-ras^{T24} gene product as immunogen. When paraffin-embedded Formalin-fixed tissue sections and the avidin-biotin complex immunoperoxidase methods were used, the RAP (RA, ras; P, peptide) MAbs clearly defined enhanced ras p21 expression in the majority of human colon and mammary carcinomas. Invasive mammary carcinomas demonstrated enhanced ras p21 expression with generally decreasing expression in carcinoma in situ, atypical hyperplasia, and non-atypical hyperplasia. The majority of all abnormal ducts and lobules from fibroadenoma and fibrocystic disease patients were negative, as well as normal mammary and colonic epithelia examined.

We have also used the RAP MAbs to define ras p21 protein expression in a spectrum of colonic disease states. Immunohistochemical analyses of individual cells within tissue sections reveal differences in ras p21 expression in colon carcinomas compared with normal colonic epithelium, benign colon tumors and inflammatory or dysplastic colon lesions. Our data suggest that ras p21 expression is correlated with depth of carcinoma within the bowel wall, and is probably a relatively late event in colon carcinogenesis. Enhanced ras p21 expression was also observed in high grade prostate and bladder carcinomas.

We have recently developed quantitative liquid competition RIAs for ras p21 using MAb Y13-259. By the use of a standard of pure recombinant ras p21, we are now able to detect p21 at fM levels, and to determine the number of molecules of p21 per cell. The concomitant use of quantitative RIAs and immunohistochemical analyses of normal, dysplastic and inflammatory disease states, as well as carcinomas, should lead to a better understanding of the role of the ras gene in the genesis of these lesions.

Alpha Transforming Growth Factors in Rodent and Human Mammary Carcinomas

Experiments are being conducted to determine the distribution and role of alpha transforming growth factors (αTGFs) in normal and malignant rodent and human mammary epithelial cells. Primary dimethylbenz(α)anthracene (DMBA) and nitrosomethylurea (NMU) rat mammary adenocarcinomas possess three

to seven-fold more biologically active and immunoreactive αTGFs than transplantable DMBA-I and NMU-II carcinomas. Moreover, a specific 5.0 kb mRNA species could be detected in the primary DMBA and NMU tumors following hybridization of poly A(+) RNA to human and mouse αTGF cDNA probes but not in the DMBA-I or NMU-II tumors. Ovariectomy produced a two to three-fold decrease in the level of αTGFs in the primary DMBA tumors which was preceeded by a loss in the expression of αTGF mRNA. Mouse mammary epithelial cells which have been transformed with a point-mutated c-Ha-ras proto-oncogene, NMuMG/ras^H cells, become resistant to the growth promoting effects of EGF because these cells have an increased capacity to synthesize αTGF mRNA. Human breast cancer cell lines are also producing αTGF and possess α TGF mRNA. In MCF-7 cells, the level of production of αTGF can be enhanced by estrogens. Elevated levels of immunoreactive αTGF and αTGF mRNA can be detected in approximately 50% of primary human breast carcinomas. There is a strong positive correlation between the presence of αTGF mRNA and the presence of functional estrogen receptors in a subset of these tumors.

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) which affect binding of the hormone to this molecule. Studies include 1) purification of the receptor from human tissue and preparation and characterization of an antibody against it; 2) examination of the nature of the subunits of the receptor, 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.

Hormones and Growth Factors in Development of Mammary Glands & Tumorigenesis

This project is designed to understand the role of hormones and growth factors in normal mammary gland development and differentiation and in development of the role 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) examine the hormonal conditions in vitro which induce production of autocrine growth factors by normal mammary tissue and breast cancer cell lines, 4) examine the effect of sialadenectomy on mammary tumor incidence and growth in mice as well as production of preneoplastic hyperplastic alveolar modules.

Augmentation of Tumor Antigen Expression

Within human breast and colon carcinoma lesions, as well as established human tumor cell lines, there exists extensive heterogeneity in the expression of defined tumor antigens as recognized by the binding of monoclonal antibodies (MAbs). This heterogeneity of tumor antigen expression can be found within defined areas of the tumor and within subcellular compartments of a single tumor cell. These studies have shown that human tumor cells have the ability to intrinsically regulate the expression of the tumor antigens. Factors such as 1) cell cycle kinetics, 2) long-term growth in vitro, 3) clonal variability, and 4) growth of tumor cells in threedimensional structures all alter the

antigen phenotype of human tumor cells. The apparent ability of human tumor cells to intrinsically modulate their tumor antigen expression provides insight into the factors contributing to the extensive antigenic heterogeneity observed in these cell populations. Studies were conducted to identify those biological response modifiers that can override the intrinsic changes in antigen expression leading to an increase in the cell surface binding of monoclonal antibodies. Recombinant human leukocyte interferon (Hu-IFN-αA) was found to increase the binding of specific MAbs to the surface of human breast and colon carcinoma cells in a dose-dependent manner. Utilizing 10-100 units Hu-IFN-αA, tumor antigen expression can be dramatically increased independent of any change in tumor cell proliferation. Cell sorter analysis revealed that the Hu-IFN-αA mediated increase in tumor antigen expression is a result of an increased amount of antigen per cell as well as the recruitment of previously antigen negative cells to become antigen positive. These studies may thus lead to: (i) a better definition of the transformed phenotype and (ii) the development of new strategies to enhance the in vivo binding of monoclonal antibodies to tumor antigens.

Localization of Human Tumors in Athymic Mice with Labeled Monoclonal Antibodies

Monoclonal antibodies B6.2 and B72.3 bind to human breast and colon tumor associated antigens. IgG from both these monoclonal antibodies was purified and F(ab')2 and Fab' fragments were prepared from the B6.2 IgG. The B72.3 IgG and B6.2 IgG and fragments were radiolabeled with I-125 and I-131 without loss of their immunoreactivity. The radiolabeled antibodies and fragments were injected into athymic mice bearing antigen positive human breast or colon tumors or an antigen-negative melanoma as a negative control. The B6.2 IgG and fragments localized specifically in the breast tumor xenografts with the IgG giving maximal activity in the tumor. The F(ab')2 fragment gave the best tumor-to-normal tissue ratios (15-20:1 for liver and spleen) due to its rapid clearance from the blood stream. The Fab' fragment cleared more rapidly than the F(ab')2 or the intact IgG with the majority of the radioactivity in the kidneys. When radiolabeled B72.3 IgG was injected into mice bearing colon carcinoma xenografts, activity in the tumor rose for the first 2 days and remained constant over the 19 day period of study. Tumor-to-normal tissue ratios rose over this period of time with ratios of approximately 18:1, for liver, spleen and kidney, at 7 days. At 19 days approximately 40% of the radiolabeled B72.3 IgG was found in the tumor. Model systems that resemble the metastatic nature of human colon carcinomas are being developed to better determine the efficacy of radiolabeled monoclonal antibodies as potential agents for radioimmunodetection and radioimmunotherapy.

Clinical Trials 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 to detect and localize colorectal carcinoma lesions using radiolabeled MAb B72.3. Parameters that will be systematically investigated concerning both the efficiency of MAb localization and the efficiency of gamma scanning of carcinoma lesions include: (a) effect of MAb dose and specific activity of radionuclide coupled MAb; (b) comparison of the use of intact IgG, $F(ab')_2$, and Fab'; (c) choice of radionuclide; (d) route of inoculation; (e) size, location, and other inherent properties of the tumor mass such as antigen content; (f) the presence of circulating antigen; (g) the presence and/or

absence of human anti-murine Ig antibodies; (h) metabolism of MAb and fragments; (i) combinations of MAbs. It is hoped that these studies will also aid in establishing a rational basis for the subsequent therapeutic or complement-mediated mechanisms, or for 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).

CELLULAR BIOCHEMISTRY SECTION (Dr. Yoon Sang Cho-Chung, Chief)

In previous work from this section it was shown that cAMP antagonizes the estrogen action and produces growth arrest of homone-dependent mammary tumors. Further, it was demonstrated that cAMP and estrogen antagonism regulates the c-ras p21 oncoprotein expression in rat mammary tumors.

In the present studies, the role of cAMP in growth control was investigated employing new site-selective cAMP analogs which far exceed in their effect the parent cAMP or the early known cAMP analogs; thus the site-selective analogs work at μM concentrations (the early known analogs required mM concentrations) and demonstrate synergism in combination. These studies demonstrated for the first time that the site-selective cAMP analogs are potent growth inhibitors of several breast and colon human cancer cells in culture as well as rat tumors in vivo, and served to elucidate the mechanism of cell growth regulation. These results suggest potential application of the site-selective cAMP analogs for the therapy of breast and colon cancers in humans.

CELL CYCLE REGULATION SECTION (Dr. William R. Kidwell, Chief)

Current hypotheses concerning the escape of tumors from normal growth controls center on the observed production of growth stimulating and inhibiting factors by tumor cells. We have examined this hypothesis by assessing growth factor and inhibitor production by normal and neoplastic human mammary cells in culture and in freshly isolated tissues. Four growth factors have been purified from these sources, including MDGFI, MDGFII, transforming growth factor alpha (TGFα), and transforming growth factorβ (TGFβ). MDGFI has been purified to apparent homogeneity. It is a Mr 62,000 acidic protein that binds to specific, high affinity receptors on mammary and other cell types. Factors that compete with MDGFI for receptor binding were detected in substantial amounts in conditioned medium from primary cultures of mammary epithelium from normal and malignant mammary tissues. Based on these assays, the production of MDGFI was estimated to be two to three times higher in malignant than normal breast cells. MDGFI was purified about 10,000 fold from human milk. The factor has an apparent molecular weight of 17,000 and pI of 4.0. It competes with EGF and TGFα for receptor binding but differs from the latter two factors in size and pI. Production of MDGII by tumor vs. normal tissues has not yet been established. Using a soft agar coloning assay, both normal and neoplastic mammary cells were found to produce large and roughly equivalent amounts of TGFβ. TGFα mRNA levels were found to correlate positively with estrogen and progesterone receptor levels in human and rodent mammary tumors and in rodents, a dramatic fall in mRNA levels was seen within three hours of ovariectomy, suggesting ovarian steroids regulate TGFα production. Both human and rodent mammary tumors contain an inhibitor of

mammary cell proliferation. The factor has been partially purified and found to be an Mr 13,000 protein with a pI of 5.0. It cross reacts with antisera against a similar factor made by bovine mammary glands. These results suggest that cell growth control is effected by positive and negative growth regulatory substances made by tumors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05190-06

PERIOD COVERED

October 1, 1985 to September 30, 1986

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)*

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Patricia Horan Hand	Chemist	LTIB, DCBD, NCI
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COOPERATING UNITS *(if any)*

P. Noguchi, Bureau of Biologics, FDA

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.3	2.3	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 managemnt of human carcinomas. These include: detection of occult tumor cells; further defining the degree of differentiation of "normal", dysplastic, and carcinoma cell populations; serum sntigens 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 epitopes on carcinoembryonic antigen (CEA) which are differentially expressed among carcinoma cell populations; (III) The generation and characterization of MAbs to proteins associated with metastatic cell populations, and (IV) The definition and characterization of breast and colon differentiation antigens.

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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 managemnt of human carcinomas. These include: detection of occult tumor cells; further defining the degree of differentiation of "normal", dysplastic, and carcinoma cell populations; serum sntigens 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 epitopes on carcinoembryonic antigen (CEA) which are differentially expressed among carcinoma cell populations; (III) The generation and characterization of MAbs to proteins associated with metastatic cell populations, and (IV) The definition and characterization of breast and colon differentiation antigens.

The high molecular weight glycoprotein (TAG-72), detected by MAb B 72.3, has been shown to have a great deal of differential expression in mammary and colon carcinoma cells as opposed to benign counterparts and a variety of normal adult tissues. Future plans are to define the biochemistry of TAG-72 and to utilize MAb B72.3 to further define the expression of this tumor antigen in the pathogenesis of both colon and breast carcinomas. Preliminary studies indicate that TAG-72 is expressed at higher levels in ovarian carcinoams than in colon or breast carcinomas. We plan to determine if TAG-72 expression is correlated with malignant vs. benign lesions, and which type of "borderline" ovarian masses express this antigen. We have recently been able to partially purify the TAG-72 antigen from human colon carcinomas. This will be used as immunogen to obtain "second generation" MAbs to TAG-72. The second generation MAbs will also provide a means for analyzing the use of cocktails of MAbs in radioimmunodetection and therapy studies, and will provide reagents to develop a double determinant RIA for TAG-72.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

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PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Oncogene Expression in Human Carcinomas

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

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Monoclonal antibodies (MAbs) of predefined specificity have been generated by utilizing a synthetic peptide reflecting amino acid positions 10-17 of the Hu-ras^{T24} gene product as immunogen. When paraffin-embedded Formalin-fixed tissue sections and the avidin-biotin complex immunoperoxidase methods were used, the RAP (RA, ras; P, peptide) MAbs clearly defined enhanced ras p21 expression in the majority of human colon and mammary carcinomas. Invasive mammary carcinomas demonstrated enhanced ras p21 expression with generally decreasing expression in carcinoma in situ, atypical hyperplasia, and non-atypical hyperplasia. The majority of all abnormal ducts and lobules from fibroadenoma and fibrocystic disease patients were negative, as well as normal mammary and colonic epithelia examined.

We have also used the RAP MAbs to define ras p21 protein expression in a spectrum of colonic disease states. Immunohistochemical analyses of individual cells within tissue sections reveal differences in ras p21 expression in colon carcinomas compared with normal colonic epithelium, benign colon tumors and inflammatory or dysplastic colon lesions. Our data suggest that ras p21 expression is correlated with depth of carcinoma within the bowel wall, and is probably a relatively late event in colon carcinogenesis. Enhanced ras p21 expression was also observed in high grade prostate and bladder carcinomas.

We have recently developed quantitative liquid competition RIAs for ras p21 using MAb Y13-259. By the use of a standard of pure recombinant ras p21, we are now able to detect p21 at fM levels, and to determine the number of molecules of p21 per cell. The concomitant use of quantitative RIAs and immunohistochemical analyses of normal, dysplastic and inflammatory disease states, as well as carcinomas, should lead to a better understanding of the role of the ras gene in the genesis of these lesions.

Major Findings:

The purpose of these studies is to generate and utilize monoclonal antibodies (MAbs) to the ras p21 oncogene and proto-oncogene products to determine the role, if any, of the ras gene in the etiology and pathogenesis of human carcinomas. To accomplish this, we first set out to develop and/or utilize MAbs to ras p21 with which we could both (a) define ras p21 expression at the single cell level, and (b) accurately quantitate ras p21.

(a) Generation of MAbs. Since the DNA sequence and thus the corresponding protein sequence of the human ras proto-onc and oncogene are known, and it has been shown that synthetic peptides can serve as immunogens, we developed MAbs to synthetic peptides reflecting amino acid positions 10-17 of the ras genome. Murine MAbs designated RAP (RAs Peptide) 1 through 5, of the IgG_{2a} subclass, were selected for reactivity to peptides reflecting the position 12 point-mutated ras p21 (Ha-ras^{T24}) and the proto-onc form (Hu-ras^{T24}). Immunoblotting experiments in which extracts were treated with $NaDodSO_4$/mercaptoethanol were used to demonstrate binding to p21.

(b) Analysis of Human Mammary Carcinomas for ras p21 Expression. Sections from 30 formalin-fixed infiltrating ductal carcinomas (IDC) from 30 patients were examined for ras gene activation with each of several RAP MAbs, with all the MAbs yielding similar results. Twenty-seven of 30 (90%) of the IDCs scored positive, while only 2 of the 21 benign breast lesions scored positive. In a separate study, invasive mammary carcinomas demonstrated enhanced ras p21 expression with generally decreasing expression in carcinoma in situ, atypical hyperplasia, and non-atypical hyperplasia. In most primary mammary carcinomas, a heterogeneity in the number of tumor cells expressing ras was observed. Several mammary tumor metastases from five patients with ras positive primary mammary tumors also showed some degree of heterogeneity of ras expression.

(c) Detection of ras p21 in Colon Carcinoma. To further define if biological correlations exist in ras p21 expression, colon carcinomas, benign colon tumors, inflammatory lesions, and normal colon from non-cancer patients were examined for reactivity with MAb RAP-1. Enhanced ras p21 expression was observed in the majority of the colon carcinomas, as opposed to none of the benign colon tumors, inflammatory colon specimens, or normal colonic tissues. Antigenic heterogeneity of ras p21 expression was also observed in primary and metastatic colon lesions. The biologic degree of malignancy of colon carcinoma correlates with the depth of tumor invasion through the bowel wall. In each specimen from colon cancer patients in which the depth of individual malignant tumor cells and glandular structures could be evaluated, enhanced Hu-ras p21 expression correlated with depth of tumor invasion.

(d) ras p21 Expression in Bladder and Prostate Carcinoma. Immunohistochemical assays have been used to localize and quantitate ras p21 in the urinary tract of bladder carcinoma patients. Increased ras p21 expression was observed in all high grade bladder carcinomas studied. This antigen may be an indicator of the malignant potential of premalignant as well as neoplastic lesions of the bladder. Immunohistochemical assays were also used to determine the presence of

ras p21 in specimens of prostate carcinoma and normal tissue. The antigen was detected in all of the 17 high grade prostate carcinomas. In contrast, no ras p21 was observed in specimens of normal prostate or benign prostatic hyperplasia.

(e) Development of a Liquid Competition RIA for ras p21. To accurately quantitate the amount of ras p21 within cells in culture or in biopsy materials, we have developed quantitative liquid competition radioimmunoassays (RIAs) for ras p21. The assays used MAb Y-259, a rat IgG MAb previously shown to immunoprecipitate the native form of both rat and human ras p21. As a standard for the sensitivity and specificity of the assays, we have obtained bacterial extracts of recombinant Hu-ras^{T24} and Hu-ras^{proto} as well as control bacterial extracts. Both ras preparations compete completely and with identical slopes in the direct binding assay. We have also obtained purified recombinant ras p21 (at least 95% pure) as a standard for quantitation. Based on the amount of purified ras p21 required for competition in this RIA, we can thus determine the moles of ras p21 per cell in virtually any sample which competes with identity in this assay. These assays, used in conjunction with specific cDNA probes to identify specific ras proto-oncogenes or point-mutated oncogenes being expressed, now provide truly quantitative analysis of ras p21 in mammalian cells to further the study of the association between ras p21 expression and transformation.

Publications:

Thor, A, Horan Hand, P., Wunderlich, D., Weeks, M., Caruso, A., Muraro, R., and Schlom, J.: Monoclonal antibodies generated to a synthetic peptide define ras gene expression at the single cell level in human colon and mammary carcinomas. In Rich, M., Hager, J., and Furmanski, P. (Eds.): RNA Tumor Viruses and Human Cancer. Boston, Martinus Nijhoff, pp. 151-167, 1985.

Horan Hand, P., Thor, A., Caruso, A., Wunderlich, D., Muraro, R., Weeks, M.O., Vilasi, V., and Schlom, J.: Comparisons of qualitative and quantitative immunoassays for ras p21. In Reisfeld, R.A., and Sell, S. (Eds.): Monoclonal Antibodies and Cancer Therapy, UCLA Symposia on Molecular and Cellular Biology, Vol. 27, New York, Alan R. Liss, Inc., pp. 23-26, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09003-04 LTIB

PERIOD COVERED

September 30, 1986 to October 1, 1986

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)*

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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	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.)*

Experiments are being conducted to determine the distribution and role of alpha transforming growth factors (αTGFs) in normal and malignant rodent and human mammary epithelial cells. Primary dimethylbenz(α)anthracene (DMBA) and nitrosomethylurea (NMU) rat mammary adenocarcinomas possess three to seven-fold more biologically active and immunoreactive αTGFs than transplantable DMBA-I and NMU-II carcinomas. Moreover, a specific 5.0 kb mRNA species could be detected in the primary DMBA and NMU tumors following hybridization of poly A(+) RNA to a human and mouse αTGF cDNA probes but not in the DMBA-I or NMU-II tumors. Ovariectomy produced a two to three-fold decrease in the level of αTGFs in the primary DMBA tumors which was preceeded by a loss in the expression of αTGF mRNA. Mouse mammary epithelial which have been transformed with a point-mutated c-Ha-*ras* proto-oncogene, NMuMG/*ras*^{H} cells, become resistant to the growth promoting effects of EGF because these cells have an increased capacity to synthesize αTGF mRNA. Human breast cancer cell lines are also producing αTGF and possess α TGF mRNA. In MCF-7 cells, the level of production of αTGF can be enhanced by estrogens. Elevated levels of immunoreactive αTGF and αTGF mRNA can be detected in approximately 50% of primary human breast carcinomas. There is a strong positive correlation between the presence of αTGF mRNA and the presence of functional estrogen receptors in a subset of these tumors.

PHS 6040 (Rev 1/84)

GPO 914-918

Major Findings:

Primary dimethylbenz(α) anthracene (DMBA) and nitrosomethylurea (NMU)-induced rat mammary tumors are estrogen (E2)-dependent, well differentiated adenocarcinomas which contain elevated levels of c-Harvey ras mRNA (primary DMBA tumors) or a point mutated c-Harvey ras proto-oncogene (primary NMU tumors). In contrast, transplantable DMBA tumors (DMBA-I) or NMU tumors (NMU-II) are E2-independent, undifferentiated carcinomas which do not possess an altered ras proto-oncogene or express elevated levels of ras mRNA. We have found that the primary DMBA or NMU tumors possess biologically active and immunoreactive αTGFs. Three to seven-fold more immunoreactive αTGF was found in the primary DMBA and NMU tumors than in the DMBA-I or NMU-II tumors which correlates with the relative level of biological activity detected in these tumors. Moreover, a specific 5.0 Kb mRNA species could be detected in the poly A (+) RNA isolated from the primary DMBA and NMU tumors which was capable of hybridizing to specific mouse and human αTGF cDNA probes following Northern blot analysis. Little or no αTGF mRNA was found in the DMBA-I or NMU-II tumors. Following ovariectomy, there was a two- to three-fold decrease in the level of immunoreactive and biologically active αTGFs in the primary DMBA tumors. This decrease was preceeded by a loss in the expression of αTGF mRNA within these tumors which could be detected within 3 hr post-ovariectomy. These results demonstrate that the levels of αTGF may be directly correlated with the degree of differentiation and E2-dependent status of these rat mammary tumors. Moreover, the levels of αTGF production may be related in some mammer to the degree or level of ras gene expression. To address this question, we have examined the level of αTGF production and αTGF mRNA expression in a mouse mammary epithelial cell line, NMuMG cells and NMuMG cells which have been transfected and transformed with a plasmid containing a human point mutated c-Harvey ras proto-oncogene, NMuMG/ras^H cells. Epidermal growth factor (EGF) or αTGF was found to stimulate the proliferation of NMuMG cells in serum-free medium or in medium containing 1% serum. In contrast, NMuMG/ras^H cells failed to respond to either growth factor under identical culture conditions. In addition, EGF or αTGF could stimulate the anchorage-independent growth (AIG) of NMuMG cells but not NMuMG/ras^H cells in soft agar as colonies. The refractoriness of the NMuMG/ras^H cells to exogenous EGF or αTGF is in part related to a three-fold reduction in the total number of cell surface EGF receptors and to an absence of a high affinity population of EGF receptor sites on these cells. Concentrated conditioned medium (CM) prepared from the NMuMG/ras^H cells were found to contain higher levels of endogenous TGF activity than CM from the NMuMG cells which could induce the AIG of NRK-49F cells in soft agar. NMuMG/ras^H CM contained two- to three-fold more biologically active and immunoreactive αTGF activity than NMuMG CM that could inhibit the binding of [^{125}I] EGF receptors in a solid-phase radioreceptor assay and that could block the binding of [^{125}I] rat αTGF to polyclonal rabbit anti-rat αTGF antibodies in a liquid-phase radioimmunoassay. Northern blot analysis of poly A(+) RNA isolated from the NMuMG and NMuMG/ras^H cells demonstrated the presence of a 5.0 Kb αTGF mRNA species in the NMuMG/ras^H cells but not in the NMuMG cells. Furthermore, a specific 2.5 Kb mRNA species which was capable of hybridizing to a human βTGF cDNA probe could be detected in both the NMuMG and NMuMG/ras^H cells. These results indicate that αTGF is at least one growth factor that is induced in

response to the presence of an activated ras proto-oncogene in mammary epithelial cells.

TGF- &s are not restricted to rodent mammary tumors since we have previously demonstrated biologically active αTGFs in the CM 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 5.0 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 αTGF levels greater than 2.5 ng/mg cell protein. Fifteen infiltrating ductal carcinomas were also examined for the presence of αTGF mRNA. Fifty-three percent (8/15) of these tumors were found to contain αTGF mRNA following Northern blot hybridization of the poly A(+) RNA with a human αTGF cDNA probe. Within this group of tumors, 75% (6/8) of the tumors were estrogen receptor positive (ER+) and progesterone receptor positive (PgR+) while the remaining 25% (2/8) were ER-/PgR-. In the TGF mRNA negative tumor group (7/15), 71% (5/7) of the tumors were ER-/PgR- while the remaining 29% (2/7) were ER+/PgR+. This strong positive correlation between the presence of αTGF mRNA and the presence of ER suggests that estrogens might regulate the production of αTGFs in vivo in those breast tumors which are estrogen responsive. Moreover, the data suggests that the presence of αTGF might be useful as an adjunct diagnostic or prognostic marker for those human breast tumors which are estrogen responsive.

Recent publications:

Zwiebel, J.A., Bano, M., Nexo, E., Salomon, D.S. and Kidwell, W.R. Partial purification of transforming growth factors from human milk. Cancer Res. 46: 933-939, 1986.

Salomon, D.S. and Perroteau, I. Growth factors in cancer and their relationship to oncogenes. Cancer Invest. 4: 43-60, 1986.

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Salomon, D.S., and Perroteau, I. Oncological aspects of growth factors. Ann. Rept. Med. Chem. (In press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09009-05 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	2.4	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Within human breast and colon carcinoma lesions, as well as established human tumor cell lines, there exists extensive heterogeneity in the expression of defined tumor antigens as recognized by the binding of monoclonal antibodies (MAbs). This heterogeneity of tumor antigen expression can be found within defined areas of the tumor and within subcellular compartments of a single tumor cell. These studies have shown that human tumor cells have the ability to intrinsically regulate the expression of the tumor antigens. Factors such as 1) cell cycle kinetics, 2) long-term growth in vitro, 3) clonal variability, and 4) growth of tumor cells in three-dimensional structures all alter the antigen phenotype of human tumor cells. The apparent ability of human tumor cells to intrinsically modulate their tumor antigen expression provides insight into the factors contributing to the extensive antigenic heterogeneity observed in these cell populations. Studies were conducted to identify those biological response modifiers that can override the intrinsic changes in antigen expression leading to an increase in the cell surface binding of monoclonal antibodies. Recombinant human leukocyte interferon (Hu-IFN-αA) was found to increase the binding of specific MAbs to the surface of human breast and colon carcinoma cells in a dose-dependent manner. Utilizing 10-100 units Hu-IFN-αA, tumor antigen expression can be dramatically increased independent of any change in tumor cell proliferation. Cell sorter analysis revealed that the Hu-IFN-αA mediated increase in tumor antigen expression is a result of an increased amount of antigen per cell as well as the recruitment of previously antigen negative cells to become antigen positive. These studies may thus lead to: (i) a better definition of the transformed phenotype and (ii) the development of new strategies to enhance the in vivo binding of monoclonal antibodies to tumor antigens.

Major Findings:

The objectives of this research project include: 1) the use of monoclonal antibodies to define antigenic heterogeneity for the expression of certain tumor antigens associated with human breast and colon carcinomas, 2) to determine the extent of antigenic modulation that occurs with specific tumor antigens, 3) to describe those intrinsic factors that are capable of modulating antigen expression, and 4) to develop strategies using biological response modifiers to overcome antigen modulation to selectively enhance the surface expression of tumor antigens.

Our studies have revealed a wide range of antigenic phenotypes among carcinoma masses. For example, ten different antigenic phenotypes emerged when 39 histologically similar infiltrating ductal carcinomas of the breast were reacted with four MAbs that recognize four different tumor antigens. One type of antigenic diversity, termed "patchwork," reveals tumor cells expressing a specific TAA directly adjacent to tumor cells negative for the same antigen. Furthermore, differential reactivity of the MAb at distinct subcellular compartments of the tumor cell at different sites within the tumor mass were also evident. For example, the TAG-72 antigen could be found as a cytoplasmic protein in one area of the tumor and as a cell surface membrane component in a different area within the same tumor mass. Similar degrees of heterogeneity of tumor antigen expression were observed within established human tumor cell lines. The MCF-7 human mammary tumor cell line was tested for the presence of TAAs using cytospin/immunoperoxidase methodology. This MCF-7 cell line contains various subpopulations of cells as defined by variability in expression of TAAs reactive with several MAbs, i.e., antigen positive MCF-7 cells were seen adjacent to cells which scored negative.

The results from several studies have shown that human carcinoma cells can intrinsically modulate the level of expression of numerous tumor antigens thereby contributing to the heterogeneity observed for these antigens. Factors such as 1) growth kinetics, 2) antigen capping and/or internalization, 3) clonality, 4) spatial, three-dimensional configuration and others have been shown to alter the level of expression of different antigens as recognized by the binding of anti-breast and colon carcinoma monoclonal antibodies. For example, the binding of MAb B6.2 was monitored on MCF-7 mammary carcinoma cells during logarithmic growth and at density-dependent arrest of cell proliferation. One day after passage, B6.2 was strongly reactive with the MCF-7 cells. Over the following seven days, the reactivity of the antibody decreased progressively and was lowest at density dependent arrest. Passage of these cells on day 7 resulted in the reappearance of the antigen, suggesting the expression of the 90 kd tumor antigen is cell cycle dependent. To investigate the relationship of antigen expression to cell cycle phase, cells at density-dependent arrest were subpassaged and monitored 24 hours later for both DNA content and cell surface antigen expression via fluorescent activated cell sorter analyses (FACS). DNA staining demonstrated that the majority of cells were in the G_0/G_1 cells express background fluorescence. In contrast, MAb B6.2 as well as MAb B38.1 were most reactive with cells in S-phase.

In light of the phenomena of "capping," "internalization," and "shedding" of lymphocyte surface antigens after exposure to MAbs, studies were underaken to determine the effect of prolonged exposure of MCF-7 mammary carcinoma cells

to MAbs B6.2 and B38.1 on cell surface expression of the reactive antigens. MCF-7 cells were incubated in the presence of monoclonal B6.2 or B38.1 for two hours, washed extensively and then refed with growth medium. Cells were then incubated for an additional 22 hours and analyzed following the addition of G/M fluorescein isothiocyanate. The membrane expression of the reactive antigens appears to be stable, which has important implications if MAbs are to be coupled with toxins which require internalization by target cells to affect cell killing.

To further characterize the antigenic heterogeneity of human mammary tumor cell populations, MCF-7 cells were cloned and ten different clones assayed for expression of cell surface TAAs. Only one MCF-7 clone, 6F1, had a parental antigenic phenotype. At least three additional phenotypes were observed among the remaining clones. To determine the stability of the antigenic phenotype of the MCF-7 clones, each cloned cell line was monitored through a four-month period and assayed during log phase growth at approximately every other passage. While some of the MCF-7 clones maintained a stable antigenic phenotype, other clones underwent dramatic changes in their cell surface antigen expression. The observation that subclones of a human tumor cell population express stable antigen phenotypes dramatically different from that of the parent suggest that the modulation of tumor antigen expression may be partially explained by the emergence and/or in vitro expansion of these cloned populations.

There exists a dichotomy in the expression of TAG-72 in carcinoma biopsies vs. established carcinoma cell lines. While a high percentage of human breast and colon carcinoma biopsies express TAG-72, only 1 of 25 breast cancer cell lines [MCF-7(one variant)] an 1 of 18 colon cancer cell lines (LS-174T) express this antigen. Furthermore, TAG-72 expression in these 2 cell lines was shown to be a property of only 1-5% of the cells. Attempts to enhance TAG-72 expression in LS-174T cells by propagation on extracellular matrix proteins such as collagen, laminin, fibronectin, or in serum-containing or serum free/ hormone supplemented media all proved unsuccessful. A pronounced increase in TAG-72 expression was observed when the LS-174T cells were grown under culture conditions which promoted three-dimensional growth. For example, LS-174T cells grown in spheroid or suspension cultures had a 2- to 7-fold higher level of expression of the TAG-72 antigen, while those grown on agar plugs demonstrated a 10-fold increase. When the LS-174T cells were injected into athymic mice, the level of TAG-72 antigen in the tumors was 100-fold higher than that measured in the cells in the culture. Thus, it seems that spatial configuration, or the cell-to-cell communication developed in three-dimensional growth, can facilitate the surface expression of this specific tumor antigen.

As shown here, antigenic heterogeneity is a well documented phenomenon of human breast, colon and other carcinomas. The presence of heterogeneity in the expression of tumor antigens on the surface of these carcinoma cells will undoubtedly be a limiting factor in the utilization of monoclonal antibodies for tumor detection and/or therapy. The studies described here present an insight into the intrinsic properties of a human tumor cell which make it capable of altering its antigenic phenotype. One can postulate that such factors also contribute to the observed antigenic heterogeneity in human carcinomas. Antigenic changes associated with growth kinetics, prolonged maintenance of cell lines in vitro and cell-to-cell communication may be relevant to manifes-

tation of the transformed and/or malignant cell type. A further understanding of the intrinsic cellular and molecular signals that modulate tumor antigen expression may help predict or control the specific antigenic phenotype of a human tumor cell population. Experimental evidence indicates that certain biological response modifiers, such as recombinant human interferon, can transmit an external stimulus which seemingly overrides the intrinsic cellular controls resulting in an increased expression of tumor antigens on the surface of human carcinoma cells. Treatment of human breast or colon carcinoma cells with Hu-IFN- αA on the expression of the respective antigens were investigated on several different cell types. Treatment of the human breast carcinoma cell, MCF-7, with 10-1,000 antiviral units of Hu-IFN-αA increased the surface expression of all three tumor antigens, CEA, TAG-72 and the B6.2-reactive 90Kd antigen. For example, CEA expression rose 34%, 94% and 169%, respectively, after a 24 hour incubation in medium containing 10, 100, 1,000 units Hu-IFN-αA/ml. The human colon carcinoma cell, WiDr, expresses both CEA and the 90Kd B6.2-specific tumor antigen, but not the B72.3 reactive TAG-72 antigen. Hu-IFN-αA treatment increased the amount of B1.1 and B6.2 bound to the WiDr cell surface, but did not initiate TAG-72 expression. For comparison, the expression of the class I major histocompatibility antigen, HLA-A, B, C, was also increased following Hu-IFN-αA treatment. It should be noted that normal human cells that do not express the tumor antigens remain negative after interferon treatment. The end result is a human tumor mass which is more homogeneous for tumor antigen expression while the surrounding normal tissues remain tumor antigen negative. This may, in turn, result in the increased binding of labeled monoclonal antibodies and thus facilitate tumor detection and therapy.

The mechanism(s) by which Hu-IFN-αA increases the expression of tumor antigens on the surface of human tumor cells is unknown. Hu-IFN-αA treatment was found to cause no change in either the size of the WiDr cells or the cellular DNA content. However, following the 24 hr treatment with 1000 antiviral units of Hu-IFN- αA, there was a substantial increase in the mean fluorescent intensity per cell as well as an increase in the number of WiDr cells expressing the tumor antigen. Therefore, it seems that Hu-IFN-αA treatment results in a recruitment of human tumor cells that did not express a detectable level of tumor antigen to become antigen positive. The overall result of the recruitment of new antigen positive cells is a human tumor cell population which is more homogeneous in its expression of a tumor associated antigen.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09012-03 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Monoclonal Antibodies to Detect Occult Carcinoma Cells

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space 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 for the detection and differentiation of carcinomas from benign cell types and other cancerous lesions.

GPO 914-918

Major Findings: A monoclonal antibody (MAb), designated B72.3, has been generated using membrane enriched fractions of a metastatic human breast carcinoma as the immunogen. Previous studies have demonstrated that the reactive antigen, a novel high molecular weight glycoprotein complex, can be detected in formalin-fixed, paraffin-embedded tissue sections of human breast and colon carcinomas, and not in variety of normal adult human tissues. We have also determined that MAb B72.3 may be used as an adjunct for diagnosis of adenocarcinoma in cytologic preparations (cell pellets and cytospins) of human effusions. Using the avidin-biotin complex method of immunoperoxidase staining MAb B72.3 was utilized to identify adenocarcinoma cells in fine needle aspiration biopsies from the lung (17/17), breast (17/21), pancreas (5/8), as well as metastases of colon (6/6) and ovary (3/3). Benign lesions of the lung, breast, pancreas, and lymph nodes were generally non-reactive. In addition, B72.3 was non-reactive with fine-needle aspirates of small-cell carcinomas of the lung, melanomas, sarcomas, and lymphomas. Thus, these data suggest that the immunocytological application of MAb B72.3 should now be considered as an adjunct in the discrimination of adenocarcinoma cells from benign lesions using the fine needle aspiration biopsy technique. In addition B72.3 may be of use in the discrimination of tumor types, particularly adenocarcinoma of the lung versus mesothelioma, small cell carcinoma vs. melanoma, sarcoma, or lymphoma.

Further analysis of TAG-72 antigen tissue distribution in benign and malignant adult and human fetal tissues have also been completed. Briefly, B72.3 was reactive with fetal gastrointestinal, pulmonary, breast and bladder epithelia; pediatric and adult normal tissues from these same sites were non-reactive. A correlation in expression between fetal tissues and malignant adult tumors was generally observed. B72.3 reactivity was noted in 100% of ovarian carcinomas (n=52) and borderline tumors (n=2) of the ovary. Benign tumors were $\leq$1% reactive in 26/27 cases. In addition, the B72.3 antigen was recently purified and utilized as an immunogen to generate a second generation of MAbs. Second generation MAbs with selective CEA reactivity have also been generated (Muraro et al.). These MAbs are currently being evaluated for their reactivities in human tissues using immunohistochemical techniques to investigate potential clinical, immunopathological, and diagnostic applications.

Currently our efforts are focused on several areas: (1) the examination of more malignant and benign effusions, especially "borderline" cases in which the diagnosis of adenocarcinoma is not absolute with B72.3 and second generation MAbs and (2) adoption of the methods employed to serve for the detection, diagnosis, and histotyping of tumor cells in fine needle aspirate biopsies.

Publications:

Szpak, C.A., Johnston, W.W., Lottich, S.C., Kufe, D., Thor, A., and Schlom, J.: Patterns of reactivity of four novel monoclonal antibodies (B72.3, DF3, B1.1, B6.2) with cells in human malignant and benign effusions: a highly selective recognition of adenocarcinoma over other neoplasms and mesothelium. Acta Cytologica 28: 356-367, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05233-05 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Purification of and Radioimmunoassays for Human Carcinoma Associated Antigens

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

David Colcher	Supv. Microbiologist	LTIB, DCBD, NCI
Peter Fuhrer	Expert	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

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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:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

A competitive radioimmunoassay (RIA) for a tumor associated antigen (termed TAG-72) identified by monoclonal antibody B72.3 has been established. The distribution of TAG-72 in human tissues has been shown to be highly specific for carcinomas with no significant reactivity to normal tissues. The RIA was used to examine sera from patients with colorectal carcinomas, other malignancies and normal sera. A mean of 2.2 units/ml of TAG-72 was found in the normal sera. When a cut-off level of 3 standard deviations above the mean level of TAG-72 found in normals is used, no patient with inflammatory disease or other benign colon diseases exhibited abnormal levels of TAG-72. Thirty-five percent of sera from advanced colon cancer patients and patients with other carcinomas were positive for TAG-72. No increase in TAG-72 reactivity was seen in normal sera or that from patients with melanomas or sarcomas. Comparison of the TAG-72 levels in sera with antigens recognized by the monoclonal antibodies currently used to screen sera of carcinoma patients clearly demonstrated that TAG-72 is different from the other antigens, and that TAG-72 can be found in some sera where no antigen is detected by the commercially available MAb RIAs.

TAG-72 has been purified from extracts of a human colon carcinoma xenograft in athymic mice using molecular sieving and antibody affinity chromatography. It has an apparent molecular weight of $\geq 10^6$ daltons, and has many properties similar to mucins.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

The purpose of these studies is 1) 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, 2) to preparatively purify these TAAs to aid in the characterization of these antigens, and 3) 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.

A competitive radioimmunoassay using a solid phase matrix was developed to enable the screening of large numbers of samples easily. Before using the competition RIA to examine serum from patients with carcinomas it was necessary to consider the effects, if any, of normal human serum on the competition RIA. This was first examined using pooled normal human sera. After normalizing binding curves with and without serum for 100% binding of the antibody to the plate in the absence of competitor, the competition curves for the TAG-72 diluted in assay buffer with and without serum were in close agreement. This validated the use of this competitive RIA for the screening of sera.

The distribution of TAG-72 in sera from patients with colorectal carcinomas and other malignancies was examined. A mean of 2.2 units/ml of TAG-72 was found in the normal sera. Some patients with advanced colon carcinomas had TAG-72 levels as much as 33 times the mean of the normals. Sera from the pre-operative colon cancer patients and from patients with an early recurrence had TAG-72 levels above the mean for the normals, but few sera had levels of greater than 3 standard deviations above the mean of the normals. None of the sera from patients with inflammatory or benign diseases had significant TAG-72 levels in their sera; their mean values were similar to those of the normals. When a cut-off level of 3 standard deviations above the mean level of TAG-72 found in normals is used, no patient with inflammatory disease (0/10) or other benign colon diseases (0/9) exhibited abnormal levels of TAG-72. A patient with local infiltrating disease and one patient classified as "early recurrence" had increased levels of TAG-72. Seven out of 20 (35%) sera from advanced colon cancer patients and sera from 7 out of 20 patients with other carcinomas were positive for TAG-72. No increase in TAG-72 reactivity was seen in normal sera or that from patients with melanomas or sarcomas.

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.

Purification of TAG-72. Monoclonal antibody B72.3 was generated by immunizing mice with extracts of a human breast tumor metastasis to the liver. Although this tumor has proven to be a rich source of TAG-72, its limited availability necessitated the identification of an alternate source of TAG-72 for purification studies. Tumors established from the LS-174T human colon carcinoma cell line had levels of TAG-72 similar to those found in the human breast tumor metastasis as measured in the competitive RIA described above. Studies were then undertaken to determine if the antigen found in LS-174T xenografts had a similar size distribution as that found in human primary and metastatic colon and breast carcinomas. Protein extracts were prepared from human colon and breast carcinomas as well as LS-174T xenografts and analyzed using Western blotting methods. The antigen detected by MAb B72.3 in all the antigen positive tumors exhibited a broad size distribution of approximately 200,000 to greater than 10^6 daltons. Large molecular weight molecules, especially mucins, are subject to shearing, resulting in the breakage of a high molecular weight form into lower molecular weight fragments. Since the extraction protocol for the antigen consisted of nitrogen cavitation followed by sonication, the range of sizes that are observed may be due to fragmentation of a high molecular weight molecule. To test this hypothesis more gentle extraction techniques were used. The resulting extracts were then run on 3-10% polyacrylamide gels and analyzed using Western blots. A higher molecular weight molecule ($\geq 10^6$) was apparent, with an absence of lower Mr forms. This indicates that the low molecular weight molecules are most likely the result of the sonication step used in the original extraction protocol. These studies have shown that the LS-174T xenograft, therefore, is an excellent source of TAG-72 and the antigen from this tumor has a molecular weight range similar to that observed in patient material. The LS-174T xenografts were therefore used in subsequent studies for the purification and characterization of TAG-72.

LS-174T colon carcinoma xenografts were homogenized and centrifuged to remove nuclei (1000 Xg) and microsomes (10,000 Xg). The post-microsomal supernatant was applied to a Sepharose CL-4B column to separate low molecular weight proteins from TAG-72. Fractions were collected and analyzed for protein and TAG-72 levels. The TAG-72 was found in the column void, as expected with an antigen of this size, while the bulk of the protein was effectively separated by the gel filtration column. The fractions containing the TAG-72 antigen were pooled and loaded onto an affinity column using the HW-65F matrix coupled with 180 mg of B72.3 IgG. Although the majority of the protein flowed through the column, most of the TAG-72 bound to the affinity matrix and was subsequently eluted using 3M NaI. Extensive studies have shown that the TAG-72 antigen can be more effectively eluted from the affinity matrix with high salt without significant loss of immunoreactivity as compared to using low pH. The eluted TAG-72 peak was then pooled, dialyzed against Tris-buffered saline, concentrated and then rechromatographed on a smaller column containing the same affinity matrix to obtain an increased degree of purification.

The purification was monitored using a competitive radioimmunoassay to quantitate TAG-72 activity, and a Lowry assay to quantitate the protein yield. The purification was also monitored by SDS-polyacrylamide gel electrophoresis to determine the purity of the TAG-72 antigen. The Coomassie stained gels demonstrate a loss in low molecular weight proteins as a function of the purification process, while the amount of high molecular weight PAS staining material increases with the purification. The PAS staining material does not stain with Coomassie Blue; this is

indicative that this material is heavily glycosylated. The size and distribution of the TAG-72 does not appear to change with purification indicating that there is no additional shearing due to the purification methods used. We were able to purify the TAG-72 antigen at least 45-fold with a recovery of approximately 14%. These values are under estimates because they are based on a comparison of the final yield of TAG-72 with the amount of TAG-72 in the post-microsomal supernatant rather than the total extract. Further studies are in progress to more accurately determine the homogeneity of the purified TAG-72 and the recovery of TAG-72 throughout the various chromatography procedures.

Publications:

Horan Hand, P., Colcher, D., Salomon, D., Ridge, J., Noguchi, P., and Schlom, J.: Spacial configuration of carcinoma cell populations influences the expression of a tumor associated glycoprotein. Cancer Res. 45: 833-840, 1985.

Horan Hand, P., Weeks, M.O., Greiner, J., Thor, A., Colcher, D., Szpak, C., Johnston, W. and Schlom, J.: Potential clinical application of a monoclonal antibody to a tumor associated glycoprotein (TAG-72). In Ceriani, R. (Ed.): Proceedings of the International Workshop on Monoclonal Antibodies and Breast Cancer. Boston, Martinus Nijnoff, 1985, pp. 108-118.

Greiner, J.W., Horan Hand, P., Wunderlich, D., and Colcher, D.: Radioimmunoassay for detection of changes in cell surface antigen expression induced by interferon. In Methods of Enzymology. New York, Academic Press, Inc., Vol. 119, pp. 682-688.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09008-05 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Localization of Human Tumors in Athymic Mice with Labeled Monoclonal Antibodies

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

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.9	2.4	.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.)*

Monoclonal antibodies B6.2 and B72.3 bind to human breast and colon tumor associated antigens. IgG from both these monoclonal antibodies was purified and F(ab')2 and Fab' fragments were prepared from the B6.2 IgG. The B72.3 IgG and B6.2 IgG and fragments were radiolabeled with I-125 and I-131 without loss of their immunoreactivity. The radiolabeled antibodies and fragments were injected into athymic mice bearing antigen positive human breast or colon tumors or an antigen-negative melanoma as a negative control. The B6.2 IgG and fragments localized specifically in the breast tumor xenografts with the IgG giving maximal activity in the tumor. The F(ab')2 fragment gave the best tumor-to-normal tissue ratios (15-20:1 for liver and spleen) due to its rapid clearance from the blood stream. The Fab' fragment cleared more rapidly than the F(ab')2 or the intact IgG with the majority of the radioactivity in the kidneys. When radiolabeled B72.3 IgG was injected into mice bearing colon carcinoma xenografts activity in the tumor rose for the first 2 days and remained constant over the 19 day period of study. Tumor-to-normal tissue ratios rose over this period of time with ratios of approximately 18:1 for liver, spleen and kidney at 7 days. At 19 days approximately 40% of the radiolabeled B72.3 IgG was found in the tumor. Model systems that resemble the metastatic nature of human colon carcinomas are being developed to better determine the efficacy of radiolabeled monoclonal antibodies as potential agents for radioimmunodetection and radioimmunotherapy.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

Tumor distribution studies with B6.2. We have shown that we can radiolabel B6.2 IgG and maintain its immunoreactivity. Athymic mice bearing the Clouser human mammary tumor were given injections of 0.1 ug of B6.2 IgG labeled with ^{125}I. The ratio of radioactivity per mg in the tumor compared to that of various tissues rose over a 4-day period and then fell at 7 days. The tumor:tissue ratios were 10:1 or greater in the liver, spleen, and kidney at Day 4. The absolute amount of ^{125}I-labeled IgG found in the tumor ranged from 2 to 15% of the injected dose, depending on tumor size. When the Clouser mammary tumor-bearing mice were given injections of ^{125}I-F(ab')$_2$ fragments of B6.2, higher tumor:tissue ratios were obtained. The tumor:tissue ratios in the liver and spleen were 15:1 to 20:1 at 96 hr. The tumor:tissue ratios were somewhat lower with blood and lungs but were still higher than those obtained using IgG. The absolute amount of the ^{125}I-F(ab')$_2$ fragments in the tumor ranged from 1 to 12% of the injected dose, depending on tumor size, with a decrease in radioactivity per mg over the 4-day period. Athymic mice bearing a human melanoma (A375), a tumor that shows no surface reactivity with B6.2 in liver cell RIAs, were used as controls for nonspecific binding of the labeled antibody or antibody fragments to tumor tissue. No preferential localization of the monoclonal antibody was observed in the control tumor; in fact, the cpm per mg in the tumor were lower than those found in many organs resulting in ratios of <1. Similarly, no localization was observed when either normal murine IgG or MOPC-21 IgG$_1$ (the same isotype as B6.2) from a murine myeloma, or their F(ab')$_2$ fragments was inoculated into athymic mice bearing Clouser mammary tumors or melanomas. The blood clearance studies following injection of ^{125}I-labeled B6.2 IgG, F(ab')$_2$ and Fab' into nude mice bearing Clouser tumors were performed. As expected, the smaller Fab' fragment cleared more rapidly than the F(ab')$_2$ fragment which in turn cleared more rapidly than the intact IgG. While molecular size probably accounts for this behavior, it is also possible that the smaller fragments are dehalogenated more rapidly, which could also accelerate clearance of ^{125}I activity from the same blood pool.

Tumor distribution studies with B72.3. Radiolocalization studies were also performed with B72.3 using athymic mice bearing human colon carcinomas (LS-174T) in comparison with a human melanoma xenograft (A375) as an antigen-negative control for nonspecific uptake of immunoglobulin. When the tumors were approximately 0.3 to 0.5 cm in diameter, the mice were given i.v. injections of approximately 1.5 uCi of ^{125}I-B72.3 IgG or ^{125}I-MOPC 21 IgG (control antibody of the same isotype). The ratio of cpm radioactivity:mg of tissue in the LS-174T tumor in comparison with that of various tissues was examined over a 7-day period. The tumor:tissue ratio rose over this period with tumor:liver, tumor:spleen, or tumor:kidney ratios of approximately 18:1 at Day 7. Tumor:blood ratios also raose during this time, resulting in ratios of 5:1 at Day 7. There was no specific uptake of ^{125}I-B72.3 IgG in any of the normal organs examined. Approximately 10-20% of the injected dose per gram reached the tumor at Day 2 post-inoculation of the radiolabeled antibody.

A major difference between the B6.2 system and the B72.3 system is that the amount of the B72.3 radiolabel at the tumor stayed constant over a longer period of time; the activity on a per gram basis then began to drop as the tumor progressed in size. The increased tumor:tissue ratios result primarily from the

clearance of labeled IgG from the blood pools. The absolute amount of radioactivity in the tumor rose over the first 2 days and then remained constant through Day 7. Athymic mice bearing melanomas (A375, a tumor line that shows no reactivity with B72.3 in live cell RIAs) were used as controls; no specific uptake of ^{125}I-B72.3 was observed in the tumors of these control animals. Similarly, no localization was observed in athymic mice bearing the colon carcinoma cell line when using ^{125}I-MOPC 21 IgG as a control antibody.

Studies were then undertaken to determine whether localization of the ^{125}I-labeled B72.3 was sufficient to detect by gamma camera scanning. Athymic mice bearing colon carcinomas or melanomas were given injections of approximately 70 uCi (approximately 5 ug) of ^{125}I-B72.3 IgG. The 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. At 48 hr the activity was still seen primarily in the tumor, with the activity in the area of the heart and lungs significantly decreased. A similar pattern was seen at 72 hr with a continuing decrease in the activity in the vital organs. The proportion of the activity found in the tumor continued to increase. Several mice bearing the control tumor, the A375 melanoma, at the same site as the LS-174T tumors were imaged at similar time points. No significant activity of the ^{125}I-B72.3 was detected in the area of the melanomas. The activity was primarily seen in the area of the heart and lungs. Even better images were obtained in the athymic mice bearing the colon carcinoma inoculated with ^{125}I-B72.3 at later time points. The background activity cleared from the mice with a $T_{1/2}$ of approximately 7.5 days. The activity in the tumor stayed essentially constant over the 19-day period studied, while the activity in the rest of the body decreased significantly. No accumulation of activity was seen in any organ. The amount of ^{125}I-B72.3 IgG in the colon carcinomas rose from 7% of the total activity in the mouse at Day 1 to approximately 40% at Day 19.

Publications:

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Greiner, J.W., Horan Hand, P., Wunderlich, D., and Colcher, D.: Radioimmunoassay for detection of changes in cell surface antigen expression induced by interferon. In Methods of Enzymology. New York, Academic Press, Inc., Vol. 119, 1986, pp. 682-688.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

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October 1, 1985 to September 30, 1986

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

Clinical Trials with Radiolabeled Antibodies

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Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Accurate detection and anatomic localization of both primary and metastatic lesions remains one of the major problems in the management of most human carcinomas. We have recently initiated clinical trials at the NIH Clinical Center to detect and localize colorectal carcinoma lesions using radiolabeled monoclonal antibody (MAb) B72.3. Parameters that will be systematically investigated concerning both the efficiency of MAb localization and the efficiency of gamma scanning of carcinoma lesions include: (a) effect of MAb dose and specific activity of radionuclide coupled MAb; (b) comparison of the use of intact IgG and $F(ab')_2$, and Fab'; (c) choice of radionuclide; (d) route of inoculation; (e) size, location, and other inherent properties of the tumor mass such as antigen content; (f) the presence of circulating antigen; (g) the presence and/or absence of human anti-murine Ig antibodies; (h) metabolism of MAb and fragments; (i) combinations of MAbs. It is hoped that these studies will also aid in establishing a rational basis for the subsequent therapeutic use of a particular MAb, either coupled to toxins, via effector cell-mediated or complement-mediated mechanisms, or using MAbs radiolabeled with one of a variety of isotopes. This latter goal can be accomplished by direct analyses of biopsy material (both tumor and normal tissues) from patients receiving radiolabeled MAb to define the "radiolocalization index" or potential "therapeutic index" (i.e., the ratio of the amount of MAb bound [via cpm] per gram of tumor tissue to that bound per gram of normal tissues).

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

We have recently initiated a clinical trial at the NIH Clinical Center using radiolabeled MAb B72.3 entitled "The Use of Radiolabeled Monoclonal Antibody to Detect Colorectal Carcinoma Metastases." This trial is being conducted in close collaboration with Dr. Paul Sugarbaker (Surgery Branch, NCI), Dr. Steven Larson (Nuclear Medicine, NIH), and Dr. G. Bryant (Pathology Branch, NCI).

The protocol for the detection of colon carcinoma metastases uses ^{131}I-MAb B72.3 and can be summarized as follows: 1. Patients are usually those who have failed conventional therapy for metastatic colon cancer and have entered the NCI Surgery Branch Protocol for the surgical resection of tumor metastases followed by administration of chemotherapy. Thus, all patients inoculated with ^{131}I-MAb B72.3 will, approximately eight days later, have metastatic tumor masses resected in addition to having multiple biopsies of normal tissues (the "normal" tissue is removed for staging purposes, i.e., to be examined for micrometastases). This permits the direct examination of biopsy specimens and thus an accurate determination as to where the MAb localizes and in what amounts. 2) Patients were also monitored for total body clearance rates via gamma scanning. 3) Serum samples will be analyzed for: (a) TAG-72 antigen levels by RIA, (b) human anti-murine antibodies via RIA (samples taken up to 3 months), (c) TAG-72 antigen/^{131}I-MAb complexes via high performance liquid chromatography (HPLC), (d) ^{131}I-MAb/human anti-murine antibody complexes via HPLC, and (e) level of cpm per ml of blood (for isotope clearance). Urine samples are obtained for 6 days post inoculation for analysis of radioactivity per ml and total cpm, i.e., ^{131}I clearance. 4) At surgery, tumor and "normal" tissues are removed and sent to Pathology. Representative samples of individual tissues are immediately weighed and analyzed for cpm of ^{131}I per gram of tissue, as well as sectioned for analysis by autoradiography. Five micron sections will be analyzed for (a) percent tumor cells of total cells present, and (b) percent of tumor cells that are positive for the TAG-72 antigen using the ABC immunoperoxidase method and MAb B72.3.

To date, 49 patients have received ^{131}I-labeled MAb B72.3. The amount of B72.3 IgG administered, the level of mCi coupled to the IgG, and specific activities (mCi^{131}I per mg MAb IgG) were varied. To date, dose levels of .2-20 mg of MAb IgG were employed. The specific activities used can be placed into 3 groups: (i) approximately 0.4 mCi of ^{131}I/mg of IgG, (ii) 2-3 mCi/mg, and (iii) 7-10 mCi/mg MAb. Clinical toxicity of any kind has not been observed in any of the patients who have received radiolabeled MAb B72.3. Gamma scans accurately identified tumor masses prior to surgery in approximately half the patients. Two points are emphasized here: (a) a "positive" represents an identification of a tumor mass <u>prior</u> to surgery and (b) anatomic localization and size of tumor mass was <u>confirmed</u> at the time of surgery. Thus localization of the tumor and tumor measurements were those obtained at surgery or immediately post operatively by gross pathology. Perhaps the most important findings of these preliminary studies are the tumor to normal tissue ratios obtained from the surgical specimens. MAb B72.3 IgG selectively localized (in terms of cpm per gram of histologically confirmed carcinoma versus cpm bound per gram of histologically confirmed normal tissue) to the vast majority of tumor masses in all patients. In most patients tumor to normal tissue MAb localization ratios ranged from 2:1 to, in most cases from 5:1 to as much as 38:1.

Publications:

Schlom, J., Colcher, D., Horan Hand, P., Greiner, J., Wunderlich, D., Weeks, M., Fisher, P.B., Noguchi, P., Pestka, S. and Kufe, D.: Monoclonal antibodies reactive with breast tumor-associated antigens. In Klein, G., Weinhouse, S. and Light, S. (Eds.): Advances in Cancer Research, New York, Academic Press, Inc., Vol. 43, 1985, pp. 143-173.

Zalutsky, M.R., Colcher, D., Kaplan, W.D. and Kufe, D.W.: Radioiodinated B6.2 monoclonal antibody: Further characterization of a potential radiopharmaceutical for the identification of breast tumors. Int. J. Nuc. Med. Biol. 12: 227-233, 1985.

Horan Hand, P., Weeks, M.O., Greiner, J., Thor, A., Colcher, D., Szpak, C., Johnston, W. and Schlom, J.: Potential clinical application of a monoclonal antibody to a tumor associated glycoprotein (TAG-72). In Ceriani, R. (Ed.): Proceedings of the International Workshop on Monoclonal Antibodies and Breast Cancer. Boston, Martinus Nijnoff, 1985, pp. 108-118.

Lundy, J., Mornex F., Keenan, A.M., Greiner, J.W. and Colcher, D.: Radioimmunodetection of human colon carcinoma xenografts in visceral organs of congenitally athymic mice. Cancer 57: 503-509, 1986.

Colcher, D., Esteban, J. and Mornex, F.: Use of monoclonal antibodies as radiopharmaceuticals for the localization of human carcinoma xenografts in athymic mice. In Colowick and Kaplan (Eds.): Methods in Enzymology. New York, Academic Press, Inc., Vol. 121, 1986, pp. 802-816.

Hayes, D.F., Zalutsky, M.R., Kaplan, W., Noska, M., Thor, A., Colcher, D. and Kufe, D.W.: Pharmacokinetics of radiolabeled monoclonal antibody B6.2 in patients with metastatic brest cancer. Cancer Res. 46: 3157-3163, 1986.

Horan Hand, P., Colcher, D., Wunderlich, D., Nuti, M., Teramoto, Y.A., Kufe, D. and Schlom, J.: Clinical implications of human mammary tumor cell heterogeneity using monoclonal antibody probes. In Reviews on Endocrine Related Cancer. ICI Pharmac. (In press).

Schlom, J., Colcher, D., Horan Hand, P., Thor, A., Greiner, J. and Weeks, M.O.: Potential clinical applications of monoclonal antibodies in colorectal and mammary cancer. In: Monoclonal Antibodies in the Diagnosis and Therapy of Cancer. Futura Publishing Company. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08274-05 LTIB

PERIOD COVERED
October 1, 1985 to September 30, 1986

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 Matarazzo	Staff Fellow	LTIB, DCBD, NCI
Ratna Biswas	Visiting Fellow	LTIB, DCBD, NCI
Claudio Dati	Visiting Fellow	LTIB, DCBD, NCI
Erika Ginsburg	Biologist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*
Dr. Kevin Lynch, University of Virginia, Charlottesville, VA
Dr. Jeffrey Bell, Cornell University, Ithaca, NY
Dr. Anthony Capuco, USDA, Beltsville, MD

LAB/BRANCH
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SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.00	2.75	.25

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

This project is designed to evaluate the nature of lactogenic hormone receptors and the factors (including other hormones) which affect binding of the hormone to this molecule. Studies include 1) purification of the receptor from human tissue and preparation and characterization of an antibody against it; 2) examination of the nature of the subunits of the receptor, 3) characterization of the nature of the interaction of Tamoxifen with membrane bound receptors related to the lactogen receptor, and 4) define 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 40kd binding unit of the prolactin (Prl) receptor, we immunopurified Prl receptors from mouse hepatic membranes. Two major bands of ~ 90 kd and 40kd were obtained. After preparative SDS-PAGE the 90kd and the 40kd regions of the gel were extracted and the receptor reconstituted Both fractions retained specific Prl binding activity. The 90kd and 4kd forms were also identified by Western blots using rabbit antimouse receptor antibody and protein cross-linking agents. We found that the 40kd unit binds actogenic hormone in the particulate as well as solubilized membrane, while the 90kd unit binds hormone in the solubilized form only. This suggests that the 40kd form is the "active" and the 90kd the "cryptic" receptor. 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 (90kd and ~ 40kd). Purified receptor was iodinated and the 2 forms separated on a G150 column. Each form was then subjected to 2D electrophoresis. The larger form has two pI's while the smaller for resolves into at least 3 distinct spots. 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 MCF-7 cells and LS174T human colon carcinoma grown in nude mice. On SDS-PAGE both tumor sources showed 3 bands with silver staining. Two major bands were at 90kd and 40-50kd. A minor band at about 60kd 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 90kd region but not in the 40-50kd region. In our continued efforts to define the relationship of membrane-bound antiestrogen binding sites (AEBS) to Prl receptors, we 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.

Publications:

Bell, J.A., Moffat, K., Vonderhaar, B.K., and Golde, D.W. Crystallization and preliminary x-ray characterization of bovine growth hormone: purification of bovine prolactin and growth hormone. J. Biol. Chem. 260: 8520-8525, 1985.

Vonderhaar, B.K., and Biswas, R. Prolactin effects and regulation of its receptors in human mammary tumor cells. In: Cellular and Molecular Biology of Experimental Mammary Cancer. D. Medina, W.R. Kidwell, G. Heppner, and E. Anderson (Eds.). Plenum Publishing Co., N.Y., (In press).

Vonderhaar, B.K. Prolactin transport, function and receptors in mammary gland development and differentiation. In: The Mammary Gland: Development, Regulation and Function. C.W. Daniel and M.C. Neville (Eds.). Plenum Publishing Co., N.Y., (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08226-10 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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 & Tumorgenesis

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

B.K. Vonderhaar	Research Chemist	LTIB, DCBD, NCI
W. Kidwell	Chief, Cell Cycle Regulation Section	LTIB, DCBD, NCI
D. Salomon	Supv. Res. Biologist	LTIB, DCBD, NCI
R. Biswas	Visiting Fellow	LTIB, DCBD, NCI
C. Dati	Visiting Fellow	LTIB, DCBD, NCI
E. Ginsburg	Biologist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. H. Nakhasi, Bureau of Biologics, FDA
Dr. Sandra Haslam, Michigan State University, East Lansing, MI

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.25	0.75

CHECK APPROPRIATE BOX(ES)

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

B

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

This project is designed to understand the role of hormones and growth factors in normal mammary gland development and differentiation and in development growth and maintenance of mammary tumors. Studies include: 1) examination of the role 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) examine the hormonal conditions in vitro which induce production of autocrine growth factors by normal mammary tissue and breast cancer cell lines, 4) examine the effect of sialadenectomy on mammary tumor incidence and growth in mice as well as production of pre-neoplastic hyperplastic alveolar modules.

GPO 914-918

Major Findings:

During the past year we established that sialadenectomy (Sx) results in temporary retardation of growth 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 form SX and control mice showed that they contained equivalent amounts of EGF-receptor competition activity/mg protein. Neither extract contained α-TGF detectable by RIA. There was 3 to 5 times more EGF (by RIA) in the Sx tumor extracts than control. Similar extracts of "normal" reduction mammoplasty human breast which was rapidly proliferating at the time of surgery contained low levels of EGF and RIA detectable α-TGF at a level comparable to levels found in breast neoplasms. By soft agar growth assay the serum of the patient was found to contain both α- and β-TGF's 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 which recognizes both EGF and α-TGF. Primary cultures of the human breast epithelium secreted αTGF into the medium at a level comparable to that of the human MCF-7 breast cancer cell line. Fibroblast cultures derived from the human breast tissue also produced low levels of both α and β-TGF's. We also found that α-TGF is as effective, and possibly more effective, that EGF in promoting lobulo-alveolar development of estrogen-progesterone primed mouse mammary glands.

We have developed an assay which allows us to assay 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.

Pubications:

Vonderhaar, B.K. and Bhattacharjee, M. The mammary gland: A model for hormonal control of differentiation and preneoplasia. In: Mihich, E. (ed): Biological Responses in Cancer: Progress Toward Potential Applications, Vol III. Plenum Publishing Co., New York, pp. 125-159, 1985.

Ginsburg, E. and Vonderhaar, B.K. Epidermal growth factor stimulates the growth of A431 tumors in athymic mice. Cancer Letters 28: 143-150, 1985.

Vonderhaar, B.K. and Nakhasi, H.L. Bifunctional activity of epidermal growth factor on α-and κ-casein gene expression in rodent mammary glands in vitro. Endocrinology, (In press).

Vonderhaar, B.K., Tang, E., Lyster, R.R. and Nascimento, M.C.S., Thyroid hormone regulation of epidermal growth factor receptor levels in mouse mammary glands. Endocrinology, (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09016-03 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Protein Synthesis During Oncogenic Transformation

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
Richard Braverman	Chemist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	0.6	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.)*

Studies have continued on the role of tropomyosin suppression in neoplastic transformation. To ascertain which, if any, components of the transformed phenotype are directly caused by suppression of tropomyosin synthesis, specific inhibition of translation of tropomyosin mRNA in intact cells was attempted by constructing an anti-sense producing vector and introducing it into NIH 3T3 cells. Successful transfectants were selected by resistance to the antibiotic, G418, and these have now been cloned. By translation of mRNA in cell-free systems and by northern blot analysis, it was established that tropomyosin suppression results from reduced levels of specific tropomyosin mRNAs. To improve the specificity of molecular probes for mouse cell tropomyosin mRNA and DNA, and to obtain more effective antisense sequences, a cDNA library derived from NIH 3T3 mRNA was produced in the lambda-gt11 expression vector. This library was screened with the TM4 cDNA probe and with anti-tropomyosin antiserum to select specific cDNA clones for the various species of tropomyosin. Thirty TM4-positive plaques were identified and purified.

GPO 914-918

Major Findings:

Studies have continued on the role of tropomyosin suppression in neoplastic transformation. Such suppression was previously found to be a common event in transformation of NIH 3T3 cells by a variety of related and unrelated retroviral oncogenes. To ascertain which, if any, components of the transformed phenotype are directly caused by suppression of tropomyosin synthesis, specific inhibition of translation of tropomyosin mRNA in intact cells was attempted by constructing an anti-sense producing vector and introducing it into NIH 3T3 cells. In the initial studies, a cDNA (clone TM4) to chicken smooth muscle tropomyosin mRNA was obtained from another laboratory. This was cloned into the pSV_2 neo vector in both the sense- and antisense- producing orientations. In collaboration with Dr. Bassin, NIH 3T3 cells have been transfected with both vectors, as well as with unmodified pSV_2 neo. Successful transfectants were selected by resistance to the antibiotic, G418, and these have now been cloned. Bulk quantities of these transfectants are now being grown to provide material for continuing studies. To determine levels of antisense production, TM4 was cloned into the pT7.1 and pT7.2 RNA transcribing vectors, permitting production of + and - strand RNA probes. These will be used in Northern blotting studies to determine relative levels of antisense and sense production in transfectants. Levels of antisense production will be correlated with levels of tropomyosin synthesis and with the appearance of neoplastic transformation.

The mechanism of suppression of tropomyosin synthesis by retroviral oncogene expression was explored. mRNAs were purified from NIH 3T3, v-Ki-ras transformed NIH 3T3 (line DT), and nontransformed cellular revertants derived from line DT (line C-11). Cell free translation products analyzed by immunoprecipitation revealed reduction in translatable mRNA for certain tropomyosins (37K and 41K) in DT cells and restoration to control or greater levels in revertants. Northern blot analysis of these mRNAs with the TM4 cDNA probe revealed reduction in some of the hybridizable tropomyosin mRNA species in DT cells and, again, restoration in revertants. It is thus established that tropomyosin suppression results from reduced levels of specific tropomyosin mRNAs. The mechanism by which expression of retroviral oncogenes causes reduced mRNA levels for specific members of the tropomyosin family of proteins is now being explored.

In order to improve the specificity of molecular probes for mouse cell tropomyosin mRNA and DNA, and to obtain more effective antisense sequences, a cDNA library derived from NIH 3T3 mRNA was produced in the lambda-gt11 expression vector. This library was screened with the TM4 cDNA probe and with anti-tropomyosin antiserum to select specific cDNA clones for the various species of tropomyosin. Thirty TM4-positive plaques were identified and purified. These will be analyzed for size, restriction sites and sequence to identify them with specific members of the tropomyosin family of proteins. Thereafter they will be used as genetic probes and for production of antisense vectors.

Publications:

Cooper, H.L., Feuerstein, N., Noda, M., and Bassin, R.H. Suppression of tropomyosin synthesis: a common biochemical feature of oncogenesis by structurally diverse retroviral oncogenes. Molec. and Cellular Biol., 5: 972-983, 1985.

Cooper, H.L. and Bassin, R.H. Biochemical changes leading to oncogenesis. In: Leukocytes and Host Defense, J. Oppenhgeim and D. Jacobs, Eds., Alan R. Liss, New York, NY, 1986, pp. 167-174.

Fagnani, R., Cooper, H.L., and Mendelsohn, J. Purification of human interleukin-2 to apparent homogeneity and partial characterization of its receptor. Analyt. Biochem. 142: 487-496, 1984.

Lal, R.B., Monos, D.S., Chused, T.M. and Cooper, H.L. Analysis of lymphocyte proteins from New Zealand black mice by two-dimensional electrophoresis. J. Immunol. 134: 2350-2356, 1985.

Fuhrer, J.P. Debiasi, F., Cooper, H.L., and Schlom, J. Analysis of ras oncogene products by two-dimensional gel electrophoresis: evidence for protein families with distinctive molecular forms. Biochim. Biophys. Acta, (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09019-02 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Cytoskeletal Proteins in Human Neoplasms

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

Herbert L. Cooper Chief, Cell. & Molc. Phys. Section LTIB, DCBD, NCI

Basudev Bhattacharya Visiting Fellow LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.8	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 synthesis and content of tropomyosins and other cytoskeletal proteins in various human tumor cell lines and tissues will be investigated in order to determine whether synthesis of these proteins is suppressed in human neoplasms as it is in NIH/3T3 cells transformed by oncogenic viruses. Tropomyosins synthesized by various tissue types will be identified by 2-dimensional polyacrylamide gel electrophoresis on the basis of molecular weight, isoelectric point, cytoskeletal localization, absence of tryptophan and immunological reactivity. Expression of tropomyosins in tumor cells of these types will then be evaluated. Synthesis of other cytoskeletal proteins will be evaluated in the same preparations. The following systems will be studied: lymphoid cells, myeloid cells, colon carcinoma, bladder carcinoma, and mammary carcinonma.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

The significance of modifications in synthesis of tropomyosin and other cytoskeletal proteins in human neoplasia is being explored. A variety of human cell lines of neoplastic and normal origin has been studied by 2-dimensional electrophoresis of whole cell proteins, proteins precipitated with antitropomyosin antisera, and proteins of cytoskeletal and other subcellular fractions. Determination of departures from normality in tropomyosin synthesis are complicated by the fact that different cell types produce varying levels of different members of the tropomyosin family of proteins. It was found that lymphoid neoplasms and normal peripheral lymphocytes do not synthesize the forms of tropomyosin which were suppressed in NIH 3T3 cells by retroviral oncogene expression. The tropomyosins synthesized by lymphocytes were apparently unaltered in neoplastic lines, but it was observed that these same tropomyosins were also expressed and were unaltered in transformed NIH 3T3 cells. It appears, therefore, that suppression of tropomyosin as a modality in neoplasia may be restricted to those cell types which normally synthesize a particular subset of tropomyosins.

In order to determine more precisely the range of variation in expression of different members of the tropomyosin gene family in cells of different lineages, the murine NMuMG line of normal mouse mammary epithelial cells was compared with NIH 3T3 fibroblasts and with NMuMG cells transformed by k-ras and polyoma. The NMuMG epithelial cells lacked expression of the prominent 41K tropomyosin typically seen in fibroblasts, but showed good expression of a 37K tropomyosin similar to one seen in fibroblasts. NMuMG cells transformed by k-ras showed no suppression of synthesis of this 37K tropomyosin, in contrast to results with NIH 3T3 fibroblasts transformed by the same oncogene. Evidently, suppression of tropomyosin synthesis in the course of retroviral oncogene expression is restricted to cells of certain lineages, notably fibroblasts.

During immunoprecipitation of tropomyosins from lysates of NIH 3T3 and NMuMG cells certain nontropomyosins coprecipitated as well. These associated proteins were specific for the antiserum used, and differed for the two types of cells. This suggests that tropomyosin may interact with different other proteins in cells of different lineages, which may be a clue to the function of tropomyosin in various types of nonmuscle cells and to the difference in effect of retroviral oncogene expression. This observation is being actively followed up.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09006-04 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Biochemical Events in Phorbol Ester Effects on Normal and Tumor Cells

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

Herbert L. Cooper	Chief, Cell. & Molec. Phys. Section	LTIB, DCBD, NCI
Richard Braverman	Chemist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.6	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Studies have continued on the unique phosphoprotein, pp17, which undergoes rapid phosphorylation in HL60 promyelocytic leukemia cells in response to treatment with phorbol ester (TPA). By tryptic peptide analysis of specific proteins recovered from 2-dimensional electrophoretic gels, the nonphosphorylated precursor (p17) of pp17 has been identified. A novel and inexpensive method for measurement of radioactivity in single proteins directly on 2-dimensional gels was devised in this laboratory by R. Braverman. Using this technique, the cellular content of p17 and the rate and extent of its phosphorylation to pp17 after TPA treatment was quantitated. p17 was found to be one of the major cytosolic proteins in HL60 cells (0.5% of total cytosolic protein). About 50% of preexisting p17 is phosphorylated within 15 minutes of addition of TPA to HL60. These findings indicate that phosphorylation of p17 is one of the most rapid, and quantitatively significant biochemical responses to TPA treatment. Since this occurs in the context of abrupt cessation of cell growth and onset of monocytoid differentiation, the possible role of p17 and pp17 in these processes is being explored. The only other phenomenon known to occur in this time frame in this system is the rapid and transitory activation of the c-fos cellular oncogene. The possibility of a functional interrelationship between these two phenomena is being explored. The possible role of p17 and its phosphorylation to pp17 in cell growth regulation in other cell types is also being explored. Preliminary results indicate increased phosphorylation of p17 in lymphoid cells which exhibit reduced cell growth rate in response to serum deprivation.

Major Findings:

Studies have continued on the unique phosphoprotein, pp17, which undergoes rapid phosphorylation in HL60 promyelocytic leukemia cells in response to treatment with phorbol ester (TPA). By tryptic peptide analysis of specific proteins recovered from 2-dimensional electrophoretic gels, the nonphosphorylated precursor (p17) of pp17 has been identified. A novel and inexpensive method for measurement of radioactivity in single proteins directly on 2-dimensional gels was devised in this laboratory by R. Braverman. Using this technique, the cellular content of p17 and the rate and extent of its phosphorylation to pp17 after TPA treatment was quantitated. p17 was found to be one of the major cytosolic proteins in HL60 cells (0.5% of total cytosolic protein). About 50% of preexisting p17 is phosphorylated within 15 minutes of addition of TPA to HL60. These findings indicate that phosphorylation of p17 is one of the most rapid, and quantitatively significant biochemical responses to TPA treatment. Since this occurs in the context of abrupt cessation of cell growth and onset of monocytoid differentiation, the possible role of p17 and pp17 in these processes is being explored. The only other phenomenon known to occur in this time frame in this system is the rapid and transitory activation of the c-fos cellular oncogene. The possibility of a functional interrelationship between these two phenomena is being explored. The possible role of p17 and its phosphorylation to pp17 in cell growth regulation in other cell types is also being explored. Preliminary results indicate increased phosphorylation of p17 in lymphoid cells which exhibit reduced cell growth rate in response to serum deprivation.

Publications:

Feuerstein, N., Nishikawa, M., and Cooper, H.L. Cell-free system studies on the phosphorylation of the 17000-20000 dalton protein induced by phorbol ester in human leukemic cells and evidence for a similar event in virally tranformed murine fibroblasts. Cancer Res. 45: 3243-3251, 1985.

Sahai, A., Feuerstein, N., Cooper, H.L. and Salomon, D.S. Effect of epidermal growth factor and 12-0-tetradecanoyl-13-acetate on phosphorylation of acidic soluble proteins in A431 epidermoid carcinoma cells. Cancer Res. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04848-14 LTIB

PERIOD COVERED

October 1, 1985 through September 30, 1986

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. Oncogenes Sect.	LTIB, DCBD, NCI
K. Yanagihara	Visiting Fellow	LTIB, DCBD, NCI
P. Tagliaferri (half time)	Visiting Fellow	LTIB, DCBD, NCI
H. Cooper	Chief, Cell. & Mole. Phys. Sect.	LTIB, DCBD, NCI
Y. Cho Chung	Chief, Cell. Biochem. Sect.	LTIB, DCBD, NCI
W. Anderson	Research Chemist	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

Dr. Claude Lechene, Harvard University, Boston, Mass.
Dr. L. Benade, American Culture Collection, Rockville, MD

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Biochemistry of Oncogenes Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Studies on revertant cells resistant to transformation by the ras gene family have been extended. Studies with the electron probe have indicated that, in every case examined, ras-transformed cells exhibit specific differences in monovalent cation transport when compared to control NIH/3T3 cells: Upon incubation in medium with 1mM ouabain or in medium lacking potassium, the loss of potassium is significantly higher in ras-transformed cells than in controls. This change most likley is responsible for the dramatically increased sensitivity of a ras-transformed cells to ouabain previously reported. Revertant cells, which contain active ras genes but are resistant to retransformation by the ras, src, and fes oncogenes, have altered phenotypes such that the loss of potassium is much less rapid. After studying the transport of potassium in 3 different revertants, we have concluded that at least 2 different mechanisms exist for generating the revertant phenotype from ras-transformed cells. Since both mechanisms result in changes in monovalent cation transport, it seems likely that this change is critical for the maintainence of the transformed phenotype.

Ouabain sensitivity of control and ras-transformed cell lines have been extended to human cells, where transformation by the ras oncogene also renders cells more susceptible to ouabain. Ouabain toxicity in human cells is pH dependant. pH effects have not been observed in mouse cells.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings

We have continued our genetic studies on transformed cells, the goal of which is to define the biochemical pathways involved in the transformation process and, ultimately, to understand the mechanism of transformation by retroviral oncogenes such as ras. Our approach has been to mutagenize populations of ras-transformed cells and to induce and isolate rare nontransformed cells called "revertants", which are resistant to retransformation. Some of these revertants should contain alterations in cellular genes involved in the transformation process. Our goal is to analyze the molecular changes which are responsible for the nontransformed phenotype in this group of revertants and thereby to elucidate the nature of the interaction of oncogene-derived proteins with cellular constituents and the mechanism of the transformation event not only in cell cultures, but also, ultimately, in tumors themselves. To date, 3 revertants resistant to the ras gene have been isolated.

During the past year, studies on revertants resistant to transformation by the ras gene have been extended. Together with Dr. Claude Lechene at Harvard University, we have examined aspects of sodium and potassium transport in control, ras-transformed, and revertant cell lines with an electron probe. This instrument measures internal concentrations of many different ions under in situ conditions. When cells are incubated either in medium lacking potassium or in growth medium containing ouabain, there are marked differences in potassium transport. All cells begin to lose potassium immediately upon exposure to ouabain or growth in medium without potassium, but the loss of potassium is much more rapid in ras-transformed cells than in contnrol NIH/3T3 cells. Three different ras-transformed cell lines and three different clones of NIH/3T3 cells displayed similar differences in potassium transport. These results support our previous studies on potassium transport using radioactive rubidium and appear to account for our previous biological studies which showed that ouabain is more toxic to ras-transformed cells than to control NIH/3T3 cells. The most critical finding for studies on the mechanism of transformation by the ras gene concerns the 3 revertant cell lines. Electron probe data show that each of the revertant cell lines has regained the slower rate of potassium efflux characteristic of parental NIH/3T3 cells, but there appear to be 2 different mechanisms of recovery. The revertant cell lines called C-11 and F-2 exhibit one pattern of potassium transport in medium with ouabain or in medium without potassium, while a third revertant line, Cl 22, shows a different pattern, one which is similar to cells with changes in sodium, potassium ATPase. We consider it highly significant that either of 2 different changes in potassium transport may be associated with resistance to the ras gene.

The nature of genes responsible for the revertant phenotype can also be defined using a molecular analysis of revertant cells. Our current after transfection of known ouabain-resistance genes into ras-transformed cells. A mouse gene recently isolated by R. Levenson confers ouabain resistance on cells and induces changes in potassium transport that seem similar to the changes noted about in our C-11 and F-2 revertants. This

gene (pRL 8) is currently under intensive investigation in our laboratory. Transfection of ouabain resistance gene from high molecular weight revertant DNA to ras-transformed cells has proven to be extremely difficult. We have developed suitable ras-transformed rat and human cell lines for use as possible recipients.

We have adapted more convenient methods with which to study the biological effects of oaubain on a larger group of transformed and control cell lines. These methods include chromium release, which measures the actual lysis of cells by ouabain; uptake of the dye crystal violet, which correlates well with cell growth; and growth in soft agar. Using these methods, we have measured the biological effects of ouabain on additional retrovral oncogene-transformed cell lines.

Studies with the human HOS cell line indicate that ras transformation of human cells is also accompanied by a change in susceptibility to ouabain, indicating that the ion transport changes associated with transformation of mouse cells are a general feature of ras tansformation. As expected, much less ouabain is required to effect ras-specific changes in human cells, indicative of the well-known higher sensitivity of human sodium, potassium ATPase as compared to the rodent form of the enzyme. In the human system, the effect of ouabain is clearly pH dependent, while in 3T3 cells this effect is not seen. We are investigating the basis for this difference.

Human tumor cell lines have been treated with ouabain and tested for growth in soft agar. While some cell lines are more sensitive than others to the effects of ouabain, a clear pattern has not yet emerged.

Publications:

Bassin, R.H. and Noda, M. Oncogene inhibition by cellular genes. In: Annual Review of Viral Oncology. Klein, G. (Ed.)., (In press).

Benade, L.E., Talbot, N., Tagliaferri, P., Hardy, C., Card., J., Noda, M., Najam, N. and Bassin, R.H. Ouabain sensitivity is linked to ras - transformation in human HOS cells: Biochem. Biophys. Res. Comm., Vol 136, pp 807-814, 1986.

Najam, N., Clair, T., Bassin, R.H., and Cho-Chung, Y.S. Cyclic AMP suppresses expression of v-ras^{H} oncogene linked to the mouse mammary tumor virus promoter. Biochem. Biophys. Res. Comm., Vol. 134, pp 436-442, 1986.

Cooper, H.L. and Bassin, R.H. Biochemical changes leading to oncogenesis. In: Leukocytes and Host Defense, J. Oppenhgeim and D. Jacobs, Eds., Alan R. Liss, New York, NY, 1986, pp. 167-174.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB08256-06 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Role of Retinoids and Hormones in Mediating Cell Growth and Differentiation

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

Wayne B. Anderson	Research Chemist	LTIB, DCBD, NCI
Thomas P. Thomas	Visiting Fellow	LTIB, DCBD, NCI

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S.P. Nissley, Metabolism Branch, NCI, NIH

LAB/BRANCH

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)

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

B

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

An early event of retinoic acid (RA) action to promote differentition of embryonal carcinoma (EC) stem cells is to sensitize the cells to cyclic AMP by elevating cyclic AMP-dependent protein kinase (cAMP-PK) activities. PCC4(RA)-1 and Nulli (RA)-1 are mutant EC cell lines which fail to differentiate in response to retinoic acid. The former, but not the latter, lacks the cellular retinoic acid-binding protein (cRABP). Treatment of Nulli (RA)-1 cells (which possess cRABP) with RA provokes an increase in cAMP-PK activities and cAMP binding, whereas RA treatment of PCC4 (RA)-1 cells (which lack cRABP) does not cause an increase in cAMP-PK activity. These results suggest that cRABP may be required to mediate the RA-induced increase in cAMP-PK activities. Other studies have demonstrated that human psoriatic fibroblasts have little, if any, R_{II} regulatory subunit when compared to R_{II} levels found with normal human fibroblasts. DEAE cellulose fractionation of extracts prepared from psoriatic fibroblasts also shows decreased type I cAMP-PK activity and the complete absence of type II kinase activity. The amount of the R_I regulatory subunit present in red blood cells obtained from psoriatic patients is significantly decreased compared to R_I levels in red cell membranes from normal subjects. These changes in R_I correlate with the severity of the disease, and indicate that determination of R_I levels in red blood cells may be used as an index to establish the presence and severity of psoriasis. Human EC (Tera 2) cells in culture offer an in vitro system to identify possible hormones and growth factors which might play a role in regulating embryonic growth and differentiation. Results indicate that Tera 2 cells have insulin-like growth factor I (IGF-I) receptors present on the cell surface. Exposure of Tera 2 cells to RA leads to differentiation to a neuronal-like cell type which exhibits enhanced calcitonin stimulation, and somatostatin inhibition, of adenylate cyclase activity. These findings suggest a possible role for IGF-I, calcitonin, and somatostatin during embryonic development.

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

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) treatment of cells. Retinoic acid induces the differentiation of PCC4 and Nulli embryonal carcinoma (EC) cells. In response to RA treatment of these undifferentiated EC cells, the levels of cAMP-PK kinase activities are elevated in the membrane fraction within 17 hours. Studies have been caried out with EC stem cell variants which are resistant to retinoid-induced differentiation. One (PCC4-RA-1) lacks the intracellular retinoic acid binding protein (cRABP). The other (Nulli-RA-1) possesses cRABP, but apparently is defective in later RA-mediated events to prevent the onset of terminal differentiation. When PCC4-RA-1 cells are treated with (RA), cAMP-PK activity is not enhanced; rather there is a decrease in cytosolic kinase activity and R_I subunit. On the other hand, Nulli-RA-1 cells (which possess cRABP) exhibit increases in both cAMP-PK activities and cAMP binding in response to RA. These results raise the possibility that cRABP mediates the enhancement of regulatory and catalytic subunits of cAMP-PK both in the membrane and cytosolic fraction of EC cells.

Since retinoids have been used with limited success to treat the proliferative skin disorder psoriasis, studies were initiated to determine cAMP-PK activities in human psoriatic fibroblasts and to determine if exposure of these cells to retinoid might sensitize psoriatic cells to cAMP. Of significance is the observation that the amount of R_{II} regulatory subunit is significantly decreased, or undetectable, in cytosol prepared from fibroblasts obtained from psoriatic patients when compared to R_{II} levels found with normal human fibroblasts. The elution profile from a DEAE cellulose column of cAMP-PK activities present in different psoriatic subjects also shows a decrease in type I kinase activity and the complete absence of type II kinase activity. Other results indicate that the amount of the R_I regulatory subunit present in red cell membranes obtained from psoriatic subjects is significantly decreased when compared to that of red cell membranes obtained from normal subjects. A significant correlation ($p<0.001$) is observed between the severity of the cutaneous manifestation of the disease and the amount of the R_I subunit present in psoriatic red cell membranes. The changes noted in the R_I and R_{II} regulatory subunits in cell types other than those thought to be specifically involved in the proliferative epidermis disorder of the disease suggest a general deficiency in cAMP-PK in human psoriasis. Due to the ease of obtaining red blood cells from patients, these results may be of value in establishing the presence, evolution, and severity of psoriasis.

Embryonal carcinoma cells have been used as a model system to identify possible hormones and growth factors which might play a role in regulating embryonic growth and differentiation. The aim of such studies is to determine the possible relationship between the embryonic factors produced by EC cells and the products of oncogenes and factors produced by certain tumor cell populations. Studies have been carried out with human teratocarcinoma (Tera 2) cells to characterize insulin-like growth factor receptors present on the cell surface. Binding of ^{125}I-IGF-I is high on Tera 2 cells. A blocking monoclonal

antibody to the type I receptor (α IR-3) completely inhibited the binding of ^{125}I-IGF-I. Further, the presence of the IGF-I receptor on Tera 2 cells was confirmed by chemically crosslinking ^{125}I-IGF-I to intact Tera 2 cells, and analyzing the solubilized radioligand-receptor complex by SDS-PAGE under reducing conditions. These results indicate that the type I, rather than the type II, is the predominant IGF receptor on confluent cultures of Tera 2 cells.

Other studies were carried out to characterize the adenylate cyclase system of Tera 2 cells. Exposure of these cells to RA leads to growth arrest and within 6 days to a differentiated, neuronal-like cell population. The cyclase of these differentiated cells exhibits a greater stimulation by calcitonin (7.5 fold) and the appearance of a somatostatin inhibitory effect. These alterations in calcitonin and somatomedin responsiveness suggest a possible regulatory role for these hormones during embryonic development, and indicate that these changes in hormonal responsiveness might serve as useful markers during early stages of human EC cell differentiation.

Publications:

Nagarajan, L., Anderson, W.B., Nissley, S.P., Rechler, M.M., and Jetten, A.M.: Production of multiplication stimulatory activity (rat IGF-II) by endoderm-like cells derived from embryonal carcinoma cells: Possible mediator of embryonic cell growth. J. Cell. Physiol. 124: 199-206, 1985.

Plet, A., Gerbaud, P., Anderson, W.B., and Evain-Brion, D.: Changes in the activity and subcellular distribution of cyclic AMP dependent protein kinases with retinoic acid-induced differentiation of embryonal carcinoma cells. Differentiation 30: 159-164, 1985.

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Plet, A., Gerbaud, P., Sherman, M.I., Anderson, W.B., Evain-Brion, D.: Retinoic acid effect on cyclic AMP-dependent protein kinases in embryonal carcinoma cells: Studies with differentiation-defective sublines. J. Cell. Physiol. 127: 341-347, 1986.

Evain-Brion, D., Raynaud, F., Plet, A., and Anderson, W.B.: Deficient cyclic AMP-dependent protein kinases in human psoriasis. 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 09015-03

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Identification of Cellular Targets of Oncogene Products

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

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Robert Bassin	Chief, Biochem. Oncogenes Sec.	LTIB, DCBD, NCI
Thomas P. Thomas	Visiting Fellow	LTIB, DCBD, NCI
Harvinder Talwar	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

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R. Gopalakrishna, Dept. of Pathology, UCLA, Los Angeles, CA

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Biochemistry of Oncogenes

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	2.7	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Studies have shown that treatment of intact cells with tumor promoters (TPA), as well as with certain growth factors and hormones, causes a rapid redistribution or stabilization (activation) of protein kinase C to the particulate fraction. Results indicate that part of the PK-C stabilized to the membrane fraction with exposure to tumor promoters may be recovered associated with nuclear-cytoskeletal components. Following treatment of NIH 3T3 cells with TPA for 20 min, PK-C activity is found tightly associated with the highly purified nuclear fraction. Immunohistochemical localization studies further support the observation that TPA treatment mediates association of PK-C to the nucleus and cytoskeleton of NIH3T3 cells. This TPA-induced redistribution of PK-C to the nuclear fraction is not observed with HL-60 cells. The different subcellular distributions of PK-C induced by TPA with different cell types may help to explain, in part, the different responses noted with various cell types with exposure to TPA. The same type of TPA-induced association of PK-C with membranes can be obtained in a reconstitution system with purified PK-C and isolated plasma membranes in the presence of TPA, Ca^{2+}, and phosphatidylserine. This TPA mediated binding is dependent upon Ca^{2+}, is time and temperature dependent, and reaches saturation at ~ 24 ng PK-C/mg PYS cell membrane protein. NIH 3T3 cells transformed with Kirsten (K_1) murine sarcoma virus have elevated membrane-associated PK-C activity and increased phosphatidylinositol (PI) metabolism relative to control 3T3 cells. In vitro studies with membranes isolated from NIH 3T3 and K_1-3T3 cells indicate PIP_2-phosphodiesterase activity is increased with K_1-SV transformation of NIH 3T3 cells. The PIP_2-PDE enzyme from K_1-3T3 cells also is more responsive to activation by GTP analogues. These results further support the suggestion for the involvement of PK-C activation (membrane association) in regulating cell proliferation, and in tumor promotion and malignant transformation, and also suggest possible modulation of this membrane signal transmission system by ras p21 protein.

Major Findings:

Although the physiological function of oncogenes and cellular homologues of retroviral oncogenes (proto-oncogenes) are still largely unknown, it is expected that the products are essential in regulating cell growth and/or cell differentiation. Studies have been aimed at characterizing regulatory interactions of the two major transmembrane signal transmission systems [adenylate cyclase and cAMP-dependent protein kinase; phosphatidylinoisitol (PI) turnover and protein kinase C (PK-C)] and to determine if these important cellular regulatory systems are targets of transformational events. A variety of hormones and growth factors interact with their cell surface receptors to stimulate PI turnover and generate diacylglycerol, a physiological activator of PK-C. Phorbol esters and other tumor promoting agents also can bind to and stimulate PK-C apparently by substituting for diacylglycerol. Other studies have established that treatment of intact cells with certain growth factors and hormones causes a rapid redistribution, or stabilization (activation) of PK-C to membranes.

Phorbol ester (TPA) treatment of intact NIH 3T3 cells causes redistribution of PK-C to the particulate fraction, predominantly to the plasma membrane. Phorbol ester treatment of NIH 3T3 cells also is observed to promote an increase in PK-C activity found associated with a cell faction containing nuclear elements and certain cytoskeletal components. In untreated cells most of the PK-C activity found associated with highly purified nucleii can be removed by chelators. However, in nucleii prepared from NIH 3T3 cells exposed to TPA for 20 min PK-C activity is tightly associated and can be recovered only by detergent extraction. Immunohistochemical localization studies with rabbit polyclonal antibody to PK-C further indicate a TPA-mediated association of PK-C to the nucleus and cytoskeleton of NIH 3T3 cells. However, treatment of HL-60 cells with TPA (which induces growth inhibition rather than growth stimulation) induces PK-C association to the plasma membrane, but not to the nuclear fraction. These results suggest that tumor promoters may induce association (activation) of PK-C with different particulate components to alter the availability of endogenous substrates. This may result in differential responses by different cell types to treatment with TPA.

To identify factors which may be involved in regulating the association of protein kinase C to membranes we have developed a reconstitution system of partially purified rat brain PK-C and membrane isolated from NIH 3T3 cells. Results indicate that the chelator-stable binding of the TPA-PK-C complex to membranes requires Ca^{2+} and phospholipids and reaches saturation at 0.1 unit (24 ng of PK-C)/mg PYS cell membrane protein. Studies with TPA treatment of cells in media with and without Ca^{2+} also show that the TPA-induced increase in membrane-associated PK-C is regulated by Ca^{2+} levels even in intact cells. TPA-stabilized membrane binding of PK-C differs in several aspects from the previously reported Ca^{2+}-induced reversible binding. The TPA-mediated association of PK-C to membranes is time and temperature dependent, while Ca^{2+}-induced reversible binding is not.

Studies also have demonstrated that interleukin 2 (IL-2) treatment of IL-2 dependent CT6 cells causes a rapid, transient redistribution (activation) of PK-C from cytosol to plasma membrane. Interleukin 2 probably acts through modulation of PI turnover to generate diacylglycerol and activate PK-C as indicated by the transient nature of PK-C membrane association. Phorbol esters also induce a

rapid phosphorylation of the antigenic epitope of the human IL-2 receptor identified by anti-Tac monoclonal antibody. Immunoprecipitated Tac derived from particulate membranes can serve as a direct substrate for purified PK-C in vitro. It remains to be established how this phosphorylation event might alter IL-2 receptor function.

Transformation of NIH 3T3 cells by Kirsten murine sarcoma virus (Ki-SV) leads to elevated levels of PK-C associated with the membrane fraction and also markedly increases the metabolism of PI as measured by the enhanced accumulation of inositol phosphates. This suggests possible alterations in the phospholipase C and PIP_2-phosphodiesterase (PIP_2-PDE) activities responsible for hydrolysis of PI and PIP_2 with K_1-SV transformation. An in vitro assay has been developed to study the hydrolysis of exogenously added [^{3}H] PI and [^{3}H] PIP_2 with membranes prepared from normal and K_1-SV transformed cells. Results indicate that PIP_2-PDE activity is enhanced with K_1-SV transformation of NIH 3T3 cells, and that the enzyme from K_1-3T3 cells is more responsive to activation by GTP analogues. Additional studies are required to determine if the ras p21 protein directly modulates PIP_2-PDE to alter the PI turnover-PK-C transmembrane signaling system.

Publications:

Farrar, W.L. and Anderson, W.B.: Interleukin 2 stimulation of protein kinase C plasma membrane association. Nature 315: 233-235, 1985.

Gopalakrishna, R., Barsky, S.H., and Anderson, W.B.: Isolation of S-100 binding proteins from brain by affinity chromatography. Biochem. Biophys. Res. Commun. 128: 1118-1124, 1985.

Gopalakrishna, R., and Anderson, W.B.: Monovalent cation-insensitive hydrophobic region on calmodulin facilitates the rapid isolation and quantitation of calmodulin free from other Ca^{2+}-dependent hydrophobic binding proteins. J. Applied Biochem. 7: 311-322, 1985.

Taguchi, M., Thomas, T.P., Anderson, W.B., and Farrar, W.L.: Direct phosphorylation of the IL-2 receptor Tac antigen epitope by protein kinase C. Biochem. Biophys. Res. Commun. 135: 239-247, 1986.

Klein, D.C., Sugden, D., Vanecek, J., Thomas, T.P., and Anderson, W.B.: Phospholipidprotein kinase C involvement in the adrenergic regulation of pinealocyte cyclic AMP. In L. Freyez, L.A. Horrocks, and G. Toffano (eds.): Phospholipids in the Nervous System: Biochemical and Molecular Pharmacology. Liviana Press, Padova, Italy, (In press).

Anderson, W.B., Thomas, T.P., Talwar, H., and Gopalakrishna, R.: Role of transmembrane signalling systems and protein kinase C in modulating cell proliferation and neoplastic transformation. In H. Eppenberger (ed.): Contributions to Oncology. Karger, Basel, Switzerland, (In press).

Thomas, T.P., Gopalakrishna, R., and Anderson, W.B.: Assessment of hormone- and tumor promoter-induced activation (membrane association) of protein kinase C in intact cells. In P.M. Conn and A.R. Means (eds.): Methods in Enzymology: Cellular Regulators. Academic Press, Orlando, FL, (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05148-07 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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
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Dr. Michael Potter, LG, DCBD, NCI, NIH
Dr. John Silver, LVD, NIAID, NIH

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.25	1.0	2.25

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.)*

BALB/c mice have previously been found to express a unique 1.7 kbp species of MMTV RNA in the lactating mamamry gland. In previous studies we have established the chromosomal locations of the endogenous MMTV genomes (designated MTV-6, MTV-8, and MTV-9). Using the feral Czech II mouse strain which lacks endogenous MMTV genomes we have genetically derived sublines which contain only MTV-6, MTV-8, or MTV-9. We have found that only the MTV-6 subline expresses the 1.7 kb MMTV RNA in lactating mammary tissue. RNA:RNA *in situ* hybridization of sections from lactating MTG mouse mammary gland show that this viral genome is expressed in discrete regions of the gland.

We have previously identified a new common integration region (designated int-3) for MMTV in MMTV (Czech II) induced mammary tumors. Recombinant clones of this region of the cellular genome have been obtained. Our recent studies have produced the following observations: (1) 6 out of 32 tumors contain a integrated viral genome at *int*-3, (2) in each case the transcriptional orientation of the viral genome is the same direction, (3) 5' to the integrated viral genome, cellular sequences are transcribed into a 2.4 kb species of RNA, (4) the *int*-3 RNA is found only in tumors with an integrated MMTV genome at this locus and is not found in normal lac mammary tissue, (5) *int*-3 is located 11cM from the H-2 locus on chromosome 17, with the gene order certromere-T-H-2-int-3.

Major Findings:

I. Endogenous MMTV in Inbred and Feral Mice.

We have previously shown that the three endogenous MMTV of BALB/c mice (designated MTV-6, MTV-8, and MTV-9) are located respectively on chromosome 16, 6, and 12. BALB/c mice express a unique 1.7 kb species of RNA corresponding to the MMTV LTR in pregnant and lactating mammary glands. To determine which endogenous MMTV genome expresses this species of RNA, we have genetically derived strains of mice containing only MTV-6, MTV-8, or MTV-9. This was accomplished by backcrossing BALB/c x Czech II F_I to Czech II mice. The Czech II mouse strain lacks endogenous MMTV genome. We found that only mice containing MTV-6 express the 1.7 kb MMTV RNA. Using in situ RNA:RNA hybridization of frozen tissue sections we found that the expression of MTV-6 occurs in discrete regions of the lactating mammary gland. Previously, we have shown that all BALB/c plasma cell tumors express the 1.7 kb MMTV RNA. Recently, lypopolysaacharide (LPS) has been shown to augment the expression of the 1.7 kb MMTV RNA in lactating mammary gland. Currently, we are using in situ RNA:RNA hybridization to determine the effect of LPS treatment on the expression of MTV-6 in the lactating mammary gland and the spleen. At least one possible outcome of these experiments is that mitogenic activation of lymphocytes makes the MTV-6 locus accessible for expression. The apparent augmentation of 1.7 kb RNA expression in LPS treated mammary gland may reflect massive lymphocyte infiltration.

II. The MMTV int Loci in Spontaneous Mammary Tumors of M. musculus musculus (Czech II) Mice.

We have previously shown that MMTV (Czech II) induced mammary tumors contain a new common integration region for MMTV which is unrelated to the MMTV int-1 and int-2 loci. This new loci designated int-3 was found to be occupied in 6 out of 32 mammary tumors. Recombinant DNA clones of this region of the cellular genome have been obtained. Using these DNA clones as probes the following has been observed: (1) Each of the viral genomes is in the same transcriptional orientation, (2) Sequences immediately 5' to the integrated viral genomes are transcribed in tumors containing a viral integration at this locus but not mammary tumors with an unaltered int-3 locus or in lactating mammary tissue. The int-3 RNA is approximately 2.4 kb in size, (3) The transcribed sequences in the int-3 locus are high conserved. Related sequences can be detected under stringent hybridization conditions in avian and several mammalian species including humans. If the architecture of the int-3 locus is like int-1 and int-2, we suspect that additional tumors will be found to contain viral insertions at the other end of the transcribed region. Currently, cDNA clones of the int-3 RNA are being obtained to further define this locus and to study the biological function of the encoded protein.

MMTV (Czech II) has recently been transmitted to BALB/c mice by foster nursing. The first mammary tumor arising in this substrain contained a viral insertion at the int-3 locus. Although more tumors will have to be examined, this result is consistent with the possibility that the different int loci will frequently be activated by a particular strain of MMTV.

We have previously shown that int-3 is located on chromosome 17. To further define its location we have examined (C3H x Czech II) F_I x Czech II backcross mice. Using

restriction fragment length polymorphisms between these two strains we found that int-3 is 11cM from the H-2 (MHC) locus, with a gene order of centromere T-H-2-int-3. Using a similar strategy we, in collaboration with John Silver, determined the location of int-2 on chromosome 7. The int-2 locus is 16 cM from Hbb (β-globin) with a gene order centromere-c-Hras-1-MTV-1-c-Hbb-int-2. This is of interest since in humans c-Hras-1 and Hbb are located on chromosome 11p and int-2 is on 11q. Each of these regions are frequently altered or deleted in human breast tumors.

Publications:

Gallahan, D., Robbins, J., Byrd, L., and Callahan, R.: The genetic stability of endogenous type B and C retroviruses in Balb/c sublines. In Potter, M. (ed.) The Balb/c Mouse-genetics and Immunology. Current Topics in Microbiology and Immunology. Springer-Verlag, Heidelburg, W. Germany, 1985, pp. 89-94.

Robbins, J.M., Gallahan, D., Hogg, E., Kozak, C., and Callahan, R.: An endogenous mouse mammary tumor virus genome common in inbred mouse strains is located on chromosome 6. J. Virol. 57: 709-713, 1986.

Escot, C., Hogg, E. and Callahan, R. Mammary tumnorigenesis in feral Mus cervicolor popaeus. J. Virol. 58: 619-625, 1986.

Callahan, R., Gallahan, D., D'Hoostelaere, L. and Potter, M. Endogenous MMTV proviral genomes in feral Mus musculus domesticus. In: The Wild Mouse in Immunology, Potter, M. (Ed.): Current Topics in Microbiology and Immunology, Springer Verlag, (In press).

Gallahan, D., Escot, C., Hogg, E. and Callahan, R. Mammary tumorigenesis in feral species of the genus Mus. In: The Wild Mouse in Immunology, Potter, M. (Ed.): Current Topics in Microbiology and Immunology, Springer Verlag, (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04829-12 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

The Identification and Characterization of Human Genes Associated with Neoplasia

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

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Charles Theillet	Visiting Fellow	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

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Dr. Rosette Lidereau, Rene Huguenin Centre, St. Cloud, France

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Oncogenetics Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.75	3.75	0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have previously described a novel family of human endogenous retroviral genomes. The nucleotide sequence of the prototype (HLM-2) has been determined. The results confirm our earlier conclusion based on blot hybridization studies that this class of viral genomes is a mosaic of sequences related to different clases of infectious retroviruses. This novel class of endogenous retroviruses is unrelated to mammalian type C retroviruses. The HLM-2 and the related HLM-25 proviral genomes have been shown to be located on respectively human chromosomes 1 and 5. Since the chimpanzee cellular genome also contains these two proviral genomes the viral integration events must have occured prior to the divergence of these species.

We have begun to determine the genetic alterations associated with breast carcinomas. This study has shown: (1) significant correlation ($p<.001$) between rare alleles of c-Hras-1 and breast cancer patients, (2) the freguent loss of a c-Hras-1 allele correlates with histopathological grade III tumors ($p<.02$), loss of estrogen and/or progesterone receptors ($p<.01$), patients which later developed a distal metastasis ($p<.05$), and (3) amplification or rearrangement of c-myc correlates ($p<.02$) with patients over 51 years of age.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

I. The Organization of MMTV related sequences in human cellular DNA

We have previously reported the isolation of recombinant DNA clones of human cellular DNA which react with the MMTV genome. Subsequently, we have shown that these sequences are located within a novel family of endogenous retroviral genes. These viral genes are a mosaic of sequences related to different classes of infectious retroviruses. Recently, the complete nucleotide sequence of the prototype HLM-2 has been determined. This particular viral genome does not appear to be biologically active, since it contains numerous translation stop signals within the coding region of the structural genes. Nucleotide sequence comparisons with those of other infectious retroviruses has confirmed our earlier conclusions based on blot hybridization data. Using a unique flanking sequence probe and human/mouse somatic cell hybrids the HLM-2 proviral genome was shown to be located on chromosome 1. A similar approach was used to define location of a related viral genome, HLM-25, to chromosome 5. Since chimpanzee cellular DNA contains the same size restriction fragments detected with the unique flanking sequence probes, we conclude that the integration of these proviral genomes into the germline occured prior to the divergence of human and chimpanzees. The LTR of the HLM-2 proviruses is reiterated 1000 times in the human genome. Most of these sequences are not associated with viral structure genes. Some of these solitary LTRs are located within or next to the Kpn-1 family of repetitive cellular sequences.

II. Genetic Alterations in human breast tumors

Recently, we have initiated a collaboration with Rosette Lidereau of the Rene Huguenin Centre in St. Cloud, France to determine whether cellular proto-oncogenes are frequently genetically altered in 104 primary human breast carcinomas. Our strategy has been to determine whether there is a correlation between the frequency of specific genetic alterations and various biochemical or histopathological properties of the tumors, the patient's history, and subsequent reoccurrences or distal metastases. We chose to focus first on the myc and ras families of protooncogenes. Examination of 20 tumors for RNA expression revealed that only cH-ras and c-myc are expressed. Not all tumors expressed these genes and their expression appears to be non coordinate. The cHras-1 gene, by restriction analysis, is polymorphic in human cellular DNA. A significant correlation ($p<.001$) between breast cancer patients and rare restriction fragment length polymorphisms was observed. Since a similar phenomenon has been observed in patients with other forms of cancer this appears not to be specific for breast cancer patients. There was no evidence of amplification or rearrangement of any of the ras proto-oncogenes. In addition, no amino acid is point mutation of c-Hras-1 or Kras-2 were observed by restriction fragment length polymorphisms in 30 breast tumor DNA. In 28% of the tumors from patients which are heterozygous for c-Hras-1 one allele was lost in the tumor DNA. This appears to be tumor specific, since the WBC DNA (where available) contained the missing allele. The loss of a c-Hras-1 allele has a significant correlation with histopathological grade III tumors ($p<.02$), estrogen and/or progesterone receptor negative tumors ($p<.01$), and patients which subseuently developed a distal metastasis ($p<.05$). whether this represents a deletion of cHras-1 or a loss of a chromosome is under investigation.

The c-myc proto-oncogene was amplified 5 to 15 fold in 32% of the carcinoma DNAs.

In 5 of 121 tumor DNAs a novel myc related restriction fragment was observed. Further analysis of one of these tumors provided evidence for a chromosomal rearrrangement 3' to the third exon. The c-myc, but not Nmyc proto-oncogene is expressed in 70% of the breast tumors examined. In some of these tumors there was no evidence of amplification. In situ RNA:RNA hybridization of frozen sections of these carcinomas demonstrated foci of silver grains over dense lymphoplasma cellular infiltrates. The amplification or rearrangement of c-myc had a significant correlation ($p<.02$) with patients over 51 years of age.

Publications

Callahan, R., Chiu, I.M., Wong, J.F.H., Tronick, S.R., Roe, B., Aaronson, S.A., and Schlom, J.: A new class of endogenous human retroviral genomes. Science 228: 1208-1211, 1985.

Callahan, R., Chiu, I., Horn, T., Ali, I., Robbins, J., Aaronson, S., and Schlom, J.: A new class of human endogenous retroviral genes. In Rich, M., Hager, J., and Furmanski, P. (Eds.): RNA Tumor Viruses and Human Cancer. Boston, Martinus Nijhoff, 1985, pp. 76-92.

Callahan, R., Ali, I., Fetherston, J., Horn, T., and Schlom, J.: The organization of mouse mammary tumor virus related sequences in human cellular DNA. In Rich, M., Hager, J.C., and Taylor-Papidimitireau, J. (Eds.): Breast Cancer: On the Edge of Discovery. Martinus Nijhoff, Boston, MA., 1985, pp. 182-189.

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Escot, C., Theillet, C., Lidereau, R., Spyratos, F., Champeme, M.H., Gest, J. and Callahan, R. Genetic alteration of the c-myc protooncogene in human primary breast carcinoma. Proc. Natl. Acad. Sci. (USA), (In press).

Lidereau, R., Escot, C., Theillet, C., Champeme, M.H., Brunet, M., Gest, J., and Callahan, R. High frequency of rare alleles of the human c-Ha-ras-1 protoncogene in breast cncer patients. J. Natl. Cancer Inst., (In press).

Theillet, C., Lidereau, R., Escot, C., Hutzell, P., Brunet, M., Gest, J., Schlom, J., and Callahan, R. The loss of a c-H-ras-1 allele and aggressive human primary breast carcinomas. Cancer Res., (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08288-04 LTIB

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October 1, 1985 to September 30, 1986

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

cDNA Cloning of N-acetyl-glucosamine β1-4 galactosyltransferase

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

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

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A specific glycosyltransferase is required for the synthesis of each of the different disaccharide linkages that are known to be present in the oligosaccharide moieties of glycoproteins and glycolipids, which are referred to as glycoconjugates. Galactosyltransferases, a subgroup of the transferases, constitute a family of enzymes which transfer galactose from UDP-gal to the non-reducing residues of oligosaccharides of various glycoconjugates as well as to monosaccharides. We have initiated the molecular cloning approach to understand the modulation of the galactoslytransferase activity, essential for generating specific cell-surface antigenic determinants. In our previous studies on the gene structural analyses of alpha-lactalbumin, a modifier or a specifier protein of galactosyltransferase, we showed that the domain of alpha-lactalbumin that interacts with galactosyltransferase is coded entirely by a separate exon. In the present studies we have cloned and sequenced a cDNA of bovine N-acetyl glucosamine β1-4galactosyltransferase and addressed the question of whether the family of galactosyltransferases have common structural and sequence features despite their varying specificities. Several sequence-related cDNA clones were isolated. cDNA insert of one set of clones encodes a protein sequence that includes the amino acid sequences of five peptides of bovine galactosyltransferase. The in vitro-translated products coded by human mRNA hybrid-selected with these sets of clones were immunologically reactive with monoclonal antibodies that recognized human but not the bovine 1-4galactosyltransferase epitopes. The sequence analyses of the cDNA clones from several sets show that the regions of the mRNA coding for either carboxyl-terminal or other domains of the protein are identical with other cDNA clones which may code for proteins that are structurally and may be functionally related to other galactosyl or galactosyltransferases.

GPO 914-918

Major Findings:

Peptide Sequences of Bovine β1-4Galactosyltransferase and Isolation and Characterization of cDNA Clones: Two mixed oligonucleotide probes, 21 and 27 bases long, were snythesized based on the sequences of two tryptic peptides isolated from purified milk bovine galactosyltransferase. These oligomers were used as hybridization probes to isolate cDNA clones for galactosyltransferase from a bovine mammary gland cDNA library of $3x10^6$ ampicillin resistant transformants constructed from lactating bovine mammary gland poly(A)$^+$RNA in the Okayama-Berg vectors. Initially about 60,000 transformants were screened. Twelve cDNA clones were identified which sorted into three groups: 4 clones hybridized to both the probes, 1 clone only to 27mer, and 7 clones only to 21mer probes. We screened 300,000 additional clones from the library and the ratio of the various types of the clones remained same as observed in the initial screening of 60,000 clones.

Identification of the cDNA of Bovineβ1-4Galactosyltransferase: The sizes of the inserts from the 1st group varied between 3.4 - 4.0 kb and their restriction endonuclease maps were identical. The longest plasmid was designated as pLbGT-1. The clone of the 2nd group, designated as pLbGT-2 contains a 4.1 kb insert and has for the most part restriction map very similar to that of pLbGT-1, except at the 5' region. The insert of pLbGT-1 hybridized with the inserts of the plasmid DNAs from the clones of 3rd group, with which only 21mer hybridizes. Subsequent DNA sequence analyses of some of the clones of the 3rd group at the site where 21mer hybridized, showed that they contain one of the primer sequences present in the mixed 21-mer but had entirely different DNA sequences compared to pLbGT-1 and pLbGT-2, which were analysed further in detail. The additional clones of pLbGT-1 and pLbGT-2 types differ from each other in length at the 5' and 3' ends, but contain common COOH-terminal coding and 3'-noncoding regions. The two sequence related cDNA clones, pLbGT-1 and pLbGT-2 were sequenced and from this and other data we conclude that pLbGT-1 encodes bovine N-acetyl-glucosamine β1-4galactosyltransferase. Several lines of evidence support this conclusion: 1) DNA Sequence Analysis: The nucleotide sequence of the 2 kb region from the 5' end of the 3.7 kb cDNA insert of pLbGT-1, encodes a protein sequence which includes the amino acid sequences of five peptides from this enzyme. The encoded protein sequence which lacks NH_2-terminal met and therefore does not encode the complete sequence of bovine 1-4galactosyltransferase, contains two potential N-glycosylation sites, ^{17}Asn-Ser-Ser and ^{44}Asn-Leu-Thr, which follow the consensus sequence Asn-X-Ser/Thr . The cDNA has, however, an unusually long 3' noncoding sequence of about 2.7 kb. The DNA sequence after the termination codon TAG, from nucleotide 988 up to nucleotide 1452, (as well in pLbGT-2), encodes, in the same open reading frame a protein of 155 residues. This DNA sequence, and the seuence in the opposite orientation between HindIII and PstI restriction sites near the 3' end, bear similarities to the Alu family sequences. These sequences, which are about 340 nucleotides long, have a strech of poly A (A_{13} at nucleotide 1310 to 1324) and are flanked by short partial repeats. Computer assisted comparison of the DNA sequences with those of the Alu family sequences further supported the conclusion that they are Alu-equivalent sequences. Comparison of the detailed restriction endonuclease

maps of pLbGT-1 and pLbGT-2 showed that a similar sequence of 3.1 kb is present in the two cDNAs, which includes 2.7 kb of the 3' noncoding region and 360 bases of the coding sequence that encodes the COOH-terminal 120 residues of the protein including the peptide corresponding to 27mer. The 3' noncoding region of both clones have, two Alu-equivalent sequences present in opposite orientations and an open reading frame encoding a protein of 155 residues. The biological significance of these sequence elements is not clear at this time. The divergent region of pLbGT-2 hybridizes with the RNA which has a size identical to that of pLbGT-1 mRNA, suggesting that this sequence is not a part of any accumlated species of a precursor mRNA which should have a size larger than that of pLbGT-1 mRNA. The exact nucleotide sequence of the region of the DNA where pLbGT-2 sequence diverges from pLbGT-1 was determined, and compared with the DNA sequence of pLbGT-1. The nucleotide sequence of pLbGT-2 was identical with the sequence of pLbGT-1 from nucleotides 624 to 2000, but distinct towards the 5' end. The nucleotide sequence near the restriction sites in the divergent region of pLbGT-2 showed no similarities with the sequence of the corresponding region of pLbGT-1. The DNA sequence at various divergent regions encode, in one of the open reading frames, peptide sequences, which are different from those of the protein encoded in the divergent part of pLbGT-1. Thus the inserts of the two plasmids encode protein sequences which have identical 120-residues of the COOH-terminal domain. These results together with the fact that the pLbGT-2 is not a single clone (since we have isolated additional clones of this type) makes it at present less likely that pLbGT-2 is a clone representing a partially spliced mRNA. The DNA sequence data shows that pLbGT-1 encodes 1-4galactosyltransferase whereas pLbGT-2 encodes a protein which is partially related to this transferase.

2) RNA Analysis by Blot Hybridization with pLbGT-1: When pLbGT-1 or its 5' end fragment was used as a hybridization probe in the RNA blot analysis, multiple mRNA sizes were detected in the total poly(A)$^+$RNA, extracted from lactating mammary glands from different species, and from the human mammary tumor cell line, MCF-7, and among them in each species was an mRNA which was as long as 4.5 kb, These RNAs were about ten fold more abundant in the lactating mammary glands of rat and mouse than in liver, which is consistent with the observation that lactating mammary glands have high levels of this enzyme. In bovine RNA two additional hybridizable bands corresponding to 2.2 and 1.8 kb were detected. In the RNA from rat and mouse lactating mammary gland, two major hybridizable bands were seen which correspond to mRNAs of about 4.2 and 2.5 kb. These results show that using bovine cDNA probes the homologous RNA sequences of multiple sizes are detected in all the other species tested.

3) Hybrid Selection and Translation: The in vitro-translated products coded by human mRNA, hybrid selected with pLbGT-1, were immunologically reactive with three different monoclonal antibodies which recognized human but not the bovine β 1-4galactosyltransferase epitopes, providing an additional evidence that pLbGT-1 contains sequences which are homologous to the human galactsyltransferase mRNA.

Common Domains among Glycosyltransferases: Evidence to date suggests that β 1-4 and α 1-3galactosyltransferases may share some common peptides. The two

blood group transferases, enzyme A (N-acetylgalactosaminyltransferase) and enzyme B(α 1-3galactosyltransferase), which transfer two different sugar moieties to the same blood group substance H, (Fuc α 1-2Gal β 1-4GlcNAc-) have also peptides in common. The differences between the encoded protein structures of pLbGT-1 and pLbGT-2 may represent transferases with some common structures and different enzymatic specificities. Currently experiments are being designed to test these possibilities.

Publications:

Qasba, P.K., and Safaya, S.K.: Structure and nucleotide sequence of the rat α-lactalbumin gene: Comparisons with the chicken egg white lysozyme gene. Nature 308: 377-380, 1984.

Narimatsu, H., Sinha, S., Brew, K., Okayama, H. and Qasba, P.K. Cloning and sequencing of cDNA of bovine N-acetlyglucosaminide β1-4galactosyltransferase. Proc. Nat. Acad. Sci. 83: 4520-4521, 1986.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05216-15 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

cAMP Receptor Protein in Cancer Growth Control

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

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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 specifically 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. Further the Site 1- and Site 2-selective analogs in combination produce synergistic enhancement of the binding to cAMP receptor protein and protein kinase activation *in vitro*.

Application of these *in vitro* findings to demonstrate cAMP analog-mediated response *in vivo*, in intact cells or tissues has been scarce. Moreover, virtually all past studies of cAMP-regulation of cell growth employed a few, early known, cAMP analogs which are weakly active for protein kinase and effective only at unphysiological high mM concentrations. The site-selective cAMP analogs which are many-fold more active for protein kinase have never been tested for their growth regulatory effect.

We, therefore, investigated the effect of site-selective cAMP analogs on the growth and morphology of several breast and colon human cancer cell lines and the *in vivo* growth of rodent mammary tumors. The analog effect on the cell growth will be correlated with the response of cAMP receptor protein present in the cancer cells. The goal of this study is to elucidate the growth regulatory mechanism of cAMP analogs which can be extrapolated to the treatment of human cancer.

GPO 914-918

Major Findings:

I. Growth arrest and morphological change of seven breast and three colon human cancer cell lines by site-selective cAMP analogs at micromolar concentrations.

Thirty-five site-selective analogs, C-2, C-6 and C-8 monosubstituted and C-2 and C-8 or C-6 and C-8 disubstituted, were tested for their growth regulatory effect on seven human breast cancer cell lines, MCF-7, MCF-7ras, T-47D, MDA-MB-231, ZR-75-1, BT-20 and HBL 100 and three colon cancer cell lines, LS 174T, WiDr and HT-29. The majority of these analogs caused an appreciable growth inhibition of all ten cancer cell lines at uM concentrations. The most potent inhibitory analogs contained chlorine atom at C-8 position, thus 8-Cl-cAMP and N^6 phenyl-8-thio Cl-phenyl-cAMP each at 50 uM concentration caused 70-80% growth inhibition of all ten cancer cell lines. N^6-benzyl-cAMP (C-6 analog) was the next potent inhibitor causing 50-60% growth inhibition at 50 uM concentration. The growth inhibition by these analogs invariably brought about a change in cell morphology. The fact that dibutyryl cAMP (10^{-3}M) which is weakly active for protein kinase did not cause growth inhibition, while the site selective analogs which are many-fold more active for protein kinase worked at uM concentrations, suggest that the growth inhibition reflects the response of protein kinase present in the cells. Thus, the site selective cAMP analogs are a potent growth inhibitor of human cancer cells and serve to elucidate the mechanism of cell growth regulation.

II. Effect of site-selective cAMP analogs on the growth of rat mammry tumors and cancer cells implanted in nude mice.

These in vivo models (nude mice, rat tumors) were used to determine the efficacy as well as toxicology of cAMP analogs. Preliminary studies demonstrate that the site-selective analogs that are most potent in the growth inhibition of human cancer cell lines (see above) were also most effective in producing tumor regression in vivo.

Publications:

Cho-Chung, Y.S.: The role of cAMP in the control of mammary tumor growth. In Hollander, V.P. (Ed.): Hormonally Responsive Tumors. New York, Academic Press, Inc., 1985, pp. 115-134.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08281-04 LTIB

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October 1, 1985 to September 30, 1986

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

The Regulatory Mechanism of Oncogene Expression

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

Y.S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBD, NCI
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Laboratory of Tumor Immunology and Biology

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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.)*

In spite of the relatively large body of information concerning the molecular structure of retrovirus oncogenes and their specific protein products, little is known about the mechanisms by which they transform cells. Moreover, proto-oncogenes, the cellular counterpart of retroviral oncogenes, have been found in normal cells but their expression is low and the mechanism for this low expression is not known. It is conceivable that substances that increase tumor development by apparently increasing cellular proliferation do so by altering the quantitative or temporal expression of cellular oncogenes. Understanding the cellular mechanisms governing the expression of both viral and cellular oncogenes would therefore provide an insight into the mechanism of neoplastic cell growth and tumor development. Occasionally, tumor cells differentiate spontaneously and then regress completely. It has been suggested that cAMP may be linked with the morphological differentiation of neoplastic cells since treatment of some tumor cells with dibutyryl cAMP, prostaglandin E_1 and inhibitors of cAMP-phosphodiesterase induces irreversible morphological differentiation. That this differentiation may be a reversion of malignancy is supported by the observation that no tumor is produced when these treated cells are inoculated into animals.

To investigate factors that affect phenotypic reversion of transformed cells, we have chosen a cell line 433 of NIH 3T3 cells containing the transforming ras gene of Ha-MuSV flanked by LTR of MMTV and clone 13-3B-4 of NIH 3T3 cells, the Ha-MuSV DNA transfectant. We also used human cancer cell lines. The goal of this study is to investigate the effect of intracellular regulatory factors, such as cyclic nucleotides, hormones, and growth factors on the expression of ras gene.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

I. Cyclic AMP Suppresses Expression of v-ras^H Oncogene Linked to the Mouse Mammary Tumor Virus Promoter

Clone 433.3 of NIH 3T3 cells is a stable carrier of the MMTV LTR:v-ras^H chimeric DNA. Only in the presence of dexamethasone (a synthetic glucocorticoid), 433.3 cells exhibit an induced level of p21 transforming protein and phenotypic transformation. $N^6,O^{2'}$-dibutyryl cAMP (DBcAMP) antagonized the effect of dexamethasone in a time - and concentration - dependent manner. DBcAMP ($5x10^{-4}$M) added 18 hr prior to the addition of dexamethasone (10^{-7}M) almost completely blocked the hormone effect: cells contained levels of p21 20% of that in the cells treated with dexamethasone alone, and formed flat, contact inhibited monolayers. On the basis of these results together with our previous data on mammary carcinomas in vivo, we postulate that cAMP may be an intracellular suppressor acting at a regulatory locus of both cellular and viral ras genes.

II. Two Classes of cAMP Analogs Synergistically Inhibit p21 ras Protein Synthesis and Phenotypic Transformation of NIH/3T3 Cells Transfected with Ha-MuSV DNA

Factors that control cellular proliferation might do so by regulating quantitative expression of viral or cellular oncogenes. Since the growth regulatory effect of cAMP is well-known, the effect of cAMP on ras gene expression was examined on Ha-MuSV-transformed 13-3B-4 cells (NIH-3T3) grown in chemically defined serum-free medium. Treatment of cells with two classes of cAMP analogs which are selective for the two different cAMP-binding sites of type II protein kinase, in combination, synergistically inhibited both p21 ras protein synthesis and phenotypic transformation. The inhibition was also demonstrated with these analogs singly but at higher concentrations. The decrease in p21 synthesis was inversely correlated with an increase in the R^{II} cAMP receptor protein, the regulatory subunit of type II protein kinase. These results suggest a role for cAMP and its receptor protein in the regulation of v-ras^H oncogene expression.

III. Growth Arrest and Suppression of p21 ras Protein Expression of Several Human and Rodent Cancer Cell Lines by Site-selective cAMP Analogs at μM Concentrations

Sixteen different cAMP analogs modified at either position C-6 or position C-8 of adenine moiety and other 16 of C-6 and C-8 disubstituted analogs were tested for their regulatory effects on the growth and p21 ras protein expression of several human breast and colon cancer cell lines and transformed rodent cell lines. All analogs when tested singly exhibited growth inhibitory effect on the rodent transformed cell lines in a concentration dependent manner, but only selected numbers of the analogs were effective in the growth inhibition of human cancer cells. In general, the C-8 analogs (Site 1-selective) required 5-10-fold less quantities than the C-6 analogs (Site 2-selective) to produce the same degree of growth inhibition. The growth inhibitory effect was synergistically enhanced when C-6 and C-8 analogs were combined. Thus, 50 μM N^6-butyryl cAMP and 5 μM 8-SCH_3 cAMP each producing 20-30% growth inhibition singly, when combined, exhibited 70-80% growth inhibition. The pairs of C-6 analogs or pairs of C-8 analogs, however, did not demonstrate synergism. The C-6 and C-8 disubstituted analogs were in general more

effective in growth inhibition of both human and rodent cancer cells as compared to the monosubstituted analogs at given concentrations. Treatment of the cells with cAMP analogs resulted in the decrease in p21 levels and increase of cAMP receptor protein R^{II} as detected by Western Blotting and immunocytochemical methods. These changes in the p21 level and R^{II} concentration were later followed by changes in the morphology of the transformed rodent cells. Cells treated with cAMP analogs exhibited a morphology characteristic of untransformed phenotype. The treatment with these cAMP analogs did not increase the intracellular cAMP levels. It is therefore concluded that the growth inhibition and suppression of ras oncogene expression by the site selective cAMP analogs are not due to increase in cellular cAMP levels but may be closely associated with the increase of the R^{II} cAMP receptor proteins.

Publications:

1. Cho-Chung, Y.S.: The role of cAMP in the control of mammary tumor growth. In Hollander, V.P. (Ed.): Hormonally Responsive Tumors. New York, Academic Press, Inc., 1985, pp. 115-134.

2. Cho-Chung, Y.S., Huang, F.L. and Kapoor, C.L.: Role of cyclic AMP in modifying the growth of mammary carcinomas: genomic regulation. In Mihich, E. (Ed.): Biological Responses in Cancer, New York, Plenum Press, Vol. 4, 1985, pp. 161-182.

3. Tagliaferri, P., Clair, T., DeBortoli, M.E., and Cho-Chung, Y.S.: Two classes of cAMP analogs synergistically inhibit p21 ras protein synthesis and phenotypic transformation of NIH/3T3 cells transfected with Ha-MuSV DNA. Biochem. Biophys. Res. Commun. 130: 1193-1200, 1985.

4. Najam, N., Clair, T., Bassin, R.H. and Cho-Chung, Y.S.: Cyclic AMP suppresses expression of v-ras^H oncogene linked to the mouse mammary tumor virus promoter. Biochem. Biophys. Res. Commun. 134: 436-442, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08280-04 LTIB

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Enhancement of Oncogene Expression and Mammary Cancer

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

Y.S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBD, NCI
T. Clair	Chemist	LTIB, DCBD, NCI
S. Ally	Guest Researcher	LTIB, DCBD, NCI

COOPERATING UNITS *(if any)*

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Dr. B.E. Haley, U. of Wyoming, Dept. of Biochemistry, Laramie, Wyoming
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2.7	2.0	0.7

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☐ (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.)*

Over twenty distinct transforming genes have been identified in the genomes of oncogenic retroviruses. Each of these oncogenes has a homologue in the chrosomal DNA of a vertebrate species. Current evidence indicates that this highly conserved set of genes may play a vital role in cell proliferation and/or differentiation. In addition, inappropriate expression of some of these genes has been implicated in the genesis of cancer. Our hypothesis is that deregulation of oncogene expression may be a possible general mechanism for the induction of neoplasia in humans. Our efforts have been concentrated on the cellular homologue of the ras gene, the oncogene carried by Harvey and Kirsten Sarcoma viruses. In this study we are investigating the role of ras gene expression in the induction of rat and human mammary carcinomas. In a study of more than 200 human breast carcinomas, we have observed elevated expression of c-ras^{H} in 70% of estrogen and progesterone receptor positive tumors and 40% of estrogen and progesterone receptor negative tumors. Whereas, an amplified or rearranged c-ras^{H} gene has not been detected in human mammary carcinomas. Thus, the mechanism by which c-ras^{H} gene expression is deregulated in these tumors remain to be determined. To study the mechanism of the enhanced c-ras^{H} gene expression, we will determine the c-ras^{H} expression in growing and growth-arrested human breast cancer cells (MCF-7), growing vs regressing rat mammary tumors, hormone-dependent vs hormone-independent tumors, and the mammary gland of rodents during normal development and chemical or viral carcinogenesis. The goal of this proposal is to provide us a fundamental basis for better understanding the mechanisms by which oncogenes involved in the neoplastic development and growth.

PHS 6040 (Rev 1/84)

GPO 914-918

Major Findings:

I. 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-ras^H 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 T_3 and T_4 stages (15 of 27 [56%]) than in tumors at T_2 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 prognosis of breast cancer.

II. THE INTERELATION BETWEEN CELLULAR ras ONCOGENE AND CYCLIC AMP IN THE GROWTH CONTROL OF MAMMARY CARCINOMA: AN ANALOGY WITH YEAST ras-cAMP SYSTEM. Exogenous dibutyryl cAMP(DBcAMP) arrests the growth of rat mammary carcinomas in vivo and human breast cancer cells (MCF-7) in culture, suggesting that abnormal cAMP regulation is involved in the growth of mammary cancer (Y.S. Cho-Chung, J. Cyclic Nucleotide Res., 6: 163, 1980). An enchanced expression of the cellular ras oncogene product, p21, has been found in mammary carcinomas of humans and rodents, and in yeast, ras proteins are controlling elements of adenylate cyclase, the cAMP synthesisizing enzyme (T.TOda et al., Cell 40: 27, 1985). We now show that in mammary carcinomas the enhanced expression of p21 accompanies an augmentation of the cAMP system demonstrating a ras gene - cAMP relation similar to that shown in yeast. Determination of p21 by Western Blotting and cAMP receptor proteins (high affinity binding proteins) by photoactivated-incorporation of 8-$N_3[^{32}P]$cAMP revealed that 53 of 77 (70%) human breast carcinomsa contained increased levels of both p21 and cAMP receptor proteins, 5 to 10 fold those of normal mammary glands. Moreover, there was a parallels increase between the p21 level and cAMP receptor level in these tumors. The major molecular species of cAMP receptor proteins were the 48K M.W. R^I and 50-52 M.W. R^{II} proteins, with dominance of the R^I over the R^{II}. In normal mammary glands, the R^I and the 50-52K M.W. R^{II} were not detected and low amounts

of 54-56K M.W. R^{II} were found. The breast tumors exhibiting the increased levels of both p21 protein and cAMP receptor proteins also contained a high cAMP level 5 to 20 fold over those of normal mammary glands. The increase of cAMP level was also found in the hormone dependent rat mammay tumors containing a high level of p21. These results suggest that quantitative activation of c-ras oncogene which leads to the abnormal cAMP regulation may have etiological significance in breast cancer.

Publications:

Cho-Chung, Y.S.: The role of cAMP in the control of mammary tumor growth. In Hollander, V.P. (Ed.): Hormonally Responsive Tumor. New York, Academic Press, Inc., pp115-134, 1985.

Cho-Chung, Y.S., Huang, F.L. and Kapoor, C.L.: Role of cyclic AMP in modifying the growth of mammary carcinomas: genomic regulation. In Mihich, E. (Ed.): Biological Responses in Cancer. New York, Plenum Publishing Corp., Vol 4, pp161-182, 1985.

Cho-Chung, Y.S., Clair, T., DeBortoli, M.E. and Tagliaferri, P.: Suppression of cellular and viral ras oncogene expression by cyclic AMP. In Neiburgs, H.E. (Ed.).: Cancer Detection and Prevention. New York, Alan R. Liss, Inc., (In press).

Cho-Chung Y.S.: Potential application of dibutryl cAMP and L-arginine in breast cancer therapy. In Kanger, S. (Ed.): Contr. Oncol. Vol 23, Basel, (In press).

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, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Hormonal Control of Growth of Normal and Neoplastic Mammary Cells

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

William R. Kidwell	Chief, Cell Cycle Regulation Sect.	LTIB, DCBD, NCI
David Salomon	Supv. Res. Biologist	LTIB, DCBD, NCI
Sanjeeva Mohanam	Visiting Fellow	LTIB, DCBD, NCI
Sue Liu	Bio. Lab. Tech	LTIB, DCBD, NCI
Brunella SanFilippo	Guest Worker	LTIB, DCBD, NCI

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2.5	0.5	2

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

B

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

Current hypotheses on the escape of tumors from normal growth controls center on the production of growth stimulating and inhibiting factors by tumor cells. We have examined this hypothesis by assessing growth factor production by normal and neoplastic human mammary cells in culture and in freshly isolated tissues. Four growth factors have been purified from these sources, including MDGFI, MDGFII, transforming growth factor alpha (TGFα), and transforming growth factorβ (TGFβ). MDGFI has been purified to apparent homogeneity. It is a Mr 62,000 acidic protein that binds to specific, high affinity receptors on mammary cells. Factors that compete with MDGFI for receptor binding were detected in conditioned medium from primary cultures of mammary epithelium from normal and malignant tissues. Based on these assays, the production of MDGFI was estimated to be two to three times higher in malignant than normal breast cells. MDGFII was purified about 10,000 fold from human milk. The factor has an apparent molecular weight of 17,000 and pI of 4.0. It competes with EGF and TGFα for receptor binding but differs from the latter two factors in size and pI. Using a soft agar cloning assay, both normal and neoplastic mammary cells were found to produce large and roughly equivalent amounts of TGFβ. TGFα mRNA levels were found to correlate positively with estrogen and progestertone receptor levels in human and rodent mammary tumors. In rodents, a dramatic fall in mRNA levels were seen within three hours of ovariectomy, suggesting ovarian steroids regulate TGFα production. Both human and rodent mammary tumors contain an inhibitor of mammary cell proliferation. The factor has been partially purified and found to be a Mr 13,000 protein with a pI of 5.0. It cross reacts with antisera against a similar factor made by bovine mammary glands. These results suggest that cell growth control is effected by positive and negative growth regulatory substances made by tumors.

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-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 1V 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 ^{125}I-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 secret about two to three times as much MDGFI as normal cells. Frozen preparations of MDGFI which were originally growth stimulatory became growth inhibitory. These observations led to the discovery of a mammary cell growth inhibitor, MCGI.

2. Mammary Cell Growth Inhibitor (MCGI). MCGI was partially purified from human milk by isoelectric focusing and HPLC chromatography. The factor is a Mr 13,000 acidic protein (pI 5.0) that is stable to S-S reducing agents. It inhibits the growth of mammary tumor cell lines at nanogram/ml levels. Enzyme-linked immunoassays indicated that MCGI is related to a similar factor isolated from bovine mammary gland by Grosse. A screening of MDGFI preparations of varying stages of purification indicated that MCGI and MDGFI co-purify through most stages. MCGI was found to interact directly with MDGFI. When ^{125}I-MCGI was incubated for 30 min with MDGFI, the chromatographic mobility of the labeled factor was found to shift on HPLC gel permeation columns. The results suggest that MDGFI might be a binding protein for MCGI.

3. Transforming Growth Factorα (TGFα). cDNA probes for TGFα mRNA were obtained from R. Dernick, Genentech. The probes were used for Northern hybridization analyses of human and rodent mammary tumor poly A+ RNA. We found that a 5 kb RNA species corresponding to full length TGF mRNA was present primarily in those tumors that were estrogen and progesterone receptor positive. Most of the tumors that were negative for receptors were also negative for TGFα mRNA. The results suggest that TGFα gene expression is controlled by ovarian steroids. Data consistent with this tenet was obtained by ovariectomy studies of rats bearing primary or transplantable mammary tumors. Within six hours of ovariectomy the TGF αmRNA levels had dropped to barely detectable levels. Similarly, it was found that estrogen addition to cultures of estrogen receptor positive human mammary cell lines resulted in an elevation in biologically and radioimmunologically active TGFα protein production.

4. Transforming Growth factorβ (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 TGFα. 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 TGFβ 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.

5. Mammary-Derived Growth Factor II (MDGFII). MDGFII was purified from human milk and a similar activity was recovered from the conditioned medium of a human mammary tumor cell line, MCF7. The factor is a Mr 17,000 protein whose biological activity is destroyed by sulfhydryl reducing agents. MDGFII interacts with TGFα (or EGF) receptors on cell membranes. The factor has been purified about 10,000 fold by isoelectric focusing and HPLC chromatography. The factor differs in size and in pI from TGFα and EGF, both of which possess similar biological activities as MDGFII.

Publications

Zwiebel, J.A., Bano, M., Nexo, E., Salomon, D.S. and Kidwell, W.R. Partial purification of transforming growth factors from human milk. Cancer Res. 46: 933-939, 1986.

Kidwell, W.R., Bano, M., Burdette, K.E., Losonczy, I., and Salomon, D.S., Mammary derived growth factors in human milk. In: Human Lactation, Jensen, R.G. and Neville, M.C. (eds). Plenum Pub. Corp., New York, pp. 209-219, 1985.

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Perroteau, I., Salomon, D.S., DeBortoli, M., Kidwell, W.R., Hazarika, P., Pardue, R., Dedman, J., and Tam, J. Immunological detection and quantitation of alpha-transforming growth factors in human breast carcinoma cells. Breast Cancer Res. Treat. In Press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08212-12 OD

PERIOD COVERED
October 1, 1985 to September 30, 1986

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.) (Neme, title, leboratory, and institute affiliation)*

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Other:	William H. Eschenfeldt	Senior Staff Fellow	OD DCBD NCI
	Marc Krug	Staff Fellow	OD DCBD NCI
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COOPERATING UNITS *(if eny)*

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3.0	3.0	0.0

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☐ (a1) Minors

☐ (a2) Interviews

B

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

A full length cDNA clone has been isolated from a human library. The messenger RNA derived from the gene is induced 20 to 80-fold upon stimulation of normal human lymphocytes with a variety of mitogens. The mRNA appears within two hours of growth stimulation and persists for at least 4 days. It appears to code either for a secreted protein or for a membrane protein. Because the mRNA is associated with growing human cells, mouse fibroblasts and mouse tissues, the data best fit a model in which the clone codes for a membrane protein required for growth.

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

Major Findings:

Upon stimulation with mitogens or antigens, lymphocytes from the peripheral circulation undergo reversible transformation into active cohorts of cells differing from one another in function, membrane characteristics and the ability to elaborate soluble factors. In this study the early events of lymphocyte activation have been examined at the molecular level. A gene has been identified which may play a fundamental role in initiating the events that lead to cell proliferation.

A cDNA library was prepared using unfractionated mRNA from normal human lymphocytes treated with Staphylococcal enterotoxin A. One clone, #536, was selected for further study based on the level at which the mRNA was induced and the kinetics of gene expression. Analysis of mRNA populations by Northern blot analysis using clone #536 as a probe revealed that the gene coded for a messenger RNA 1300 nucleotides long. The mRNA was present at low levels in resting lymphocytes but increased 20 to 80-fold within 24 hours of mitogen stimulation. The response was virtually identical with Staphylococcal enterotoxins A and B, phyto hemagglutinin, concanavalin A or, to a lesser extent, exfoliatin serving as the mitogen. After induction with Staphylococcal enterotoxin A, a marked increase in mRNA was observed within 2 hours, with further increases at 6 and 24 hours. Thereafter, the levels remained constant for 3 days. Since DNA synthesis in activated normal human lymphocytes begins 18 to 20 hours after induction and continues for days, the behavior of the mRNA is consistent with a role in cell proliferation.

The 1.3 Kb messenger RNA was also found in HTLV-1 infected cells.

Messenger RNA from mouse tissues and mouse fibroblasts was examined with the human cloned probe. A homologous mRNA, similar in size to the human mRNA, was found in liver, kidney and spleen; heart and brain were apparently negative. Clone #536 mRNA was also present in actively growing mouse 3T3 cells but decreased dramatically when serum was withdrawn. On release from serum deprivation, however, the levels increased for 12 hours with marked changes occurring in 1 to 2 hours. Although the serum deprived 3T3 cell is only superficially quiescent, whereas the resting lymphocyte is deeply so, both cells responded to growth stimulation with an early and significant increase in clone #536 mRNA.

Human genomic DNA was screened with the cloned cDNA by the Southern blot technique. The results suggested that either the gene has multiple introns, or that there are several identical or closely related genes.

Clone #536 was subcloned and sequenced with the aim of identifying its product. The 340 base pairs that comprised the clone were examined and compared with the gene and cDNA sequences in the Genbank. Homology with the human oncogene, C-fos, and yeast cytochrome C was demonstrated but the match was not convincing in either case. Since there were no other candidates, the conclusion was drawn that the #536 sequence was unique.

Using a technique devised in this laboratory, the location of the cloned fragment within the mRNA was determined. The approach makes use of specific

oligomers to create substrates for ribonuclease H. The data proved conclusively that the cloned fragment was located 150 bases from the 5'-end of the 1.3 Kb mRNA and suggested that the region probably included coding sequences. Translation of those sequences using the most probable reading frame indicated that the putative protein contained many basic amino acids. A full length clone has recently been isolated from a vector-primer type library derived from SV40 transformed human fibroblasts. Extended sequence information will be forthcoming shortly and might aid in identification.

A synthetic capped mRNA was generated from the full length clone. Translation of the mRNA in vitro together with analysis of the protein might provide clues to the function of the gene. The protein will also be used to prepare an antibody.

The 1.3 Kb mRNA can be found in human myeloma cells. Because these cells are inherently virtually ribonuclease-free, it was possible to fractionate cytoplasm into membrane bound and free polysomes. When mRNA was isolated from both components and analyzed, sequences capable of hybridizing to clone #536 DNA were found almost exclusively in the membrane bound fraction. This means that the cloned material codes either for a secreted product or for a membrane-associated protein. Since clone #536 mRNA is not lymphocyte specific, but is associated with growing cells, in general, we favor the hypothesis that it codes for a membrane protein required for growth.

In other projects, we have developed methods for the efficient reverse transcription of giant (7.5 Kb) mRNAs into full length cDNAs.

Recombinant DNA constructs for studying mRNA turnover have been made.

Publications:

Krug, M. S., and Berger, S. L.: A Micromethod for Measuring the Molar Concentration of Polyadenylated RNA in the Presence of Ribosomal RNA. Anal. Biochem. 153: 315-323, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05526-18 OD

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

P53: A Common Protein in Embryonic Differentiation and In Cellular Transformation.

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

PI:	Peter T. Mora	Supervisory Chemist	OD DCBD NCI
Other:	V. W. McFarland	Chemist	OD DCBD NCI
	C. Dale Smith	Visiting Fellow	OD DCBD NCI
	M. John Louis	Visiting Fellow	OD DCBD NCI

COOPERATING UNITS *(if any)* Pierre May, Fogarty Scholar; Frank Hetrick, University of Maryland; Anton Jetten, National Institute of Environmental Health Sciences; Janice Chou, MCB, NICHHD, NIH; Johng S. Rhim, DCE, NCI, NIH

LAB/BRANCH

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are interested in the nuclear phosphoprotein p53 and of its possible role in cellular transformation to tumorigencity, and also in the normal cellular processes of differentiation and cell division. We have detected a specific 2kb p53 mRNA in chicken and also in fish cells. The p53 mRNA decreases drastically in the developing chicken embryo, similar to a decrease in the developing mouse embryo. These declines are apparently due to differences in the processing of the mRNA, and account for the decline of the p53 protein during embryonal differentiation.

The complexing of the p53 with the SV40 T antigen in SV40 transformed cells is conditional; it depends on the type of cell studied, and it is unrelated to the transformation to the tumorigenic state. There is no correlation of the tumorigenic phenotype and of the steady state levels and of the half life of p53 protein and of mRNA, when well matched normal and derivative tumorigenic cells are compared. Our findings emphasize the postulated involvement of this evolutionarily well preserved protein in the general cellular processes of cell division and of differentiation, rather than in the tumorigenic transformation per se.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

1. Changes In The p53 In Embryogenesis. Using a full length p53 cDNA clone as a probe the steady state level of the p53 mRNA was found to decrease drastically during the embryonal development of the mouse. This is in accord with our earlier finding on the decline of the metabolically labelled p53 protein during embryogenesis. We have now shown a similar decline by quantitating the the p53 protein steady state levels in Western blots. We also detected and quantitated a specific p53 mRNA of the chicken in Northern blots, using a chicken p53 cDNA (work with Pierre and Evelyn May). This mRNA is 2 kb in size, similar to the mammalian p53 mRNA molecules, and it cross-reacts with the mammalian cDNA probes. Having the convenient and inexpensive chicken egg system available, we extended our study and found that the p53 mRNA also decreases dramatically during the embryonal development of the chicken. Results of mRNA half life and nuclear run on RNA analyses indicated that differences in mRNA processing is causing this decrease. This is consistent with what we (and others) also observed in other systems of cell differentiation, such as in the induced differentiation in tissue culture of the F9 embryonal carcinoma cells upon the addition of retinoic acid. We also detected a specific 2kb mRNA for p53 in mRNA extracts from fish (epidemial papilloma of carp and salmon embryo) cells (work with F. Hettrick. Thus the p53 is an evolutionary well conserved nuclear phosphoprotein, consistent with its postulated role in normal cellular processes, such as cell division and cellular differentiation.

2. p53 in Cellular Transformation. Numerous clones of mouse tumor cells, which all came from various tumors upon spontaneous transformation of essentially non-tumorigenic cloned cells (fibroblasts) were obtained in our laboratory in the past. The non-tumorigenic parent clones and the derivative highly tumorigenic clones (sarcoma) were compared biochemically. There was no increse in the steady state level of p53 protein or in the half life of the p53. Neither was an increase in the steady state mRNA levels for p53, or for a large number of cellular protooncogenes related to the RNA tumor viruses. Our finding on p53 protein level was rapidly confirmed by the group of A. B. Pardee at Boston and extended to the transformations of other mouse cells (Balb 3T3) with sarcoma viruses and with benzo(a)pyrane. Our findings are significant as they show that in the spontaneous transformation and in the in vivo selection for the tumorigenic cells, similar to the natural evolution of the tumors, regulation of the p53, or of the recognized protooncogenes, is apparently not a factor.

We have found in the past that in the spontaneously transformed Balb 3T12 cells, when further transformed by SV40, there is no complexing between the p53 and the T antigen, apparently due to other cellular factors, such as the enzymes involved in phosphorylation of these proteins. We extended these studies and found that in normal human cell lines, which were transformed by SV40 and thereby immortalized, there is also no complexing. These cell lines included human placenta and ammion epithelial lines, and also human fibroblast lines, transformed with tsA and ori$^-$ mutants, and also with wild type SV40 (work with J. Chou). Similarly, no complexing occurs in TAg$^+$ human epidermal keratinocytes infected with AD12-SV40, even when the cells are further transformed by the Kirsten sarcoma virus and thereby acquired tumorigenicity.

However, when the neoplastic conversion for tumorigenicity was by a chemical carcinogen (MNNG) the T antigen became complexed to the p53 (work with J. S. Rhim and S. Aaronson). These findings together with findings by others using SV40 deletion mutants, established that the complexing of the p53 to the T antigen provides no specific contribution to the cellular transformation to tumorigenicity.

Publications:

Thathamangalam, U., Chandrasekaran, K., Hoffman, J. C., McFarland, V. W., Parrott, C., Simmons, D. T., and Mora, P. T.: The Transformation-Related Protein p53 is Not Bound to the T Antigen in SV40-Transformed Balb 3T12 Cells. Virology, 1986, in press.

Mora, P. T., Parrott, C. L., Baksi, K., and McFarland, V. W.: Immunologic Selection of SV40 T-Antigen-Negative Tumor Cells Which Arise by Excision of Early SV40 DNA. J. Virology, 1986, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00941-30 OD

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic and Other Factors Affecting Marrow Transplantation in Irradiated Inbred Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: Delta E. Uphoff Research Biologist OD DCBD NCI

COOPERATING UNITS *(if any)*
None

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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) Minors
☐ (a2) Interviews

B/D

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

The investigation of physical factors affecting success of bone marrow transplantation experiments was continued and included both radiobiological effects of the exposure and subtle changes in physical factors that alter survival of irradiated inbred mice with and without marrow grafts. Basic concepts of radiation biology were demonstrated to be invalid and physical factors considered to have insignificant effects were demonstrated to be critical for the reproducibility of data. Subtle changes in the quality of X-ray resulted in significant differences in results. Exposure-rate effects and exposure-rate in combination with dose reduction by absorption resulted in altered patterns of repair of lethal and sublethal cellular damage and were a function of the direction of the exposure. The heterogeneous nature of the hematopoietic system which consists of circulating cells, cells encased in skeletal bone and in soft tissue of the spleen, thymus, and lymph nodes and the potential for a single normal or neoplastic cell to repopulate the system required greater emphasis on physical conditions of the radiation than has been considered necessary in the past. More precise methods of determining the physical parameters as they affect the experimental data should be devised. In addition, the practice of converting calibrated exposures in air to absorbed tissue doses by using inaccurate and inappropriate coefficients of absorption should be abandoned in all cases where the exposure to a specific tissue or organ can not be measured directly. There is a need for a radical change in the way physical factors are viewed when dealing with biological systems.

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

Project Description

Objectives:

To investigate genetic and physical factors influencing the success of bone marrow replacement following administration of toxic levels of ionizing irradiation and/or chemotherapeutic agents used for cancer treatment. The ultimate goal was to devise methods of prevention and control of lethal graft vs. host reaction while utilizing immunotherapeutic benefits of less severe reactions, however, it became apparent that to obtain consistently reproducible results a reevaluation of some universally accepted concepts and practices used for irradiating recipients was required.

Methods Employed:

To test the validity of general radiobiological concepts and practices as applied to treatment of the hematopoietic system required an experimental system capable of testing single variables. In addition recognition of the complexities of this heterogeneous organ system was necessary for interpretation of the data. A mouse cannot be assumed to be a container of H2O nor a suspension of chemical elements as was the case when it was recommended that calibrations in air expressed as roentgens (R) be converted to an absorbed dose by use of an absorption coefficient and expressed in rads or Grays (Gy). In these experiments the target system consisted of circulating cells, cells encased in skeletal bone and cells of the soft tissue organs of the spleen, thymus and lymph nodes. To fully appreciate the experimental observations in a biological system consisting of complex interactions required the use of intact animals of known genetic origin. Over the years an experimental system capable of detecting 25R difference in exposure to X-rays was developed using inbred strains and substrains raised under conventional conditions in a closed colony set up for this purpose. All substrains were traceable to a single pair of genetically defined parents and foster parents to minimize variables introduced through the maternal influence since maternal influences were readily detected using both irradiation alone and with marrow transplantation. All experiments involved more than one inbred strain to differentiate data reflecting unique strain differences from data involving general phenomena. Both X-rays and gamma rays from cesium-137 were used as radiation sources.

Major Findings:

The "spectrum" of the wavelength of X-rays has been defined in terms of filtration for biological purposes while the "quality" of X-rays has been defined in terms of half value layer (HVL). These imprecise but easily obtained measurements were considered adequate for the insensitive biological systems which were thought to be incapable of responding differently to X-rays of different wavelengths. The first physical factor recognized to affect survival of lethally irradiated, marrow inoculated mice was the quality of the X-rays produced by 2 different machines operated with the same filters and adjusted to have the same HVL. Another method of altering the detectable quality of X-rays without changing the calibration of the machine was to operate the

machine with and without the auxillary line voltage regulator. This fact made possible the investigation of this phenomenon without requiring duplicate machines i.e., in these experiments the single variable was the auxillary line voltage regulator. Applying the observations on mice to the test of the wave form of the currents used to produce the X-rays revealed a change in the amplitude of the wave form when the machine was operated with and without the auxillary line voltage regulator. Changes in the amplitude of the wave form should have changed the output of the X-ray machine, however, no change was detected using 2 different methods of calibration. This problem remains unresolved but deserves further investigation since it offers a possible explanation for why apparently duplicate experiments conducted in different laboratories produce different results. It also demonstrates the sensitivity to which some biological systems may be developed. In addition some laboratories use conditions of "maximum backscatter" to reduce the exposure time, under these conditions the wavelength of the primary radiation differs from that of secondary ionization produced by the backscatter. Biological systems that detect changes in the wavelength of the primary radiation will certainly respond differentily to the greater differences in the secondary ionization compared to the primary ionization, i.e., maximum backscatter should not be used to irradiate animals when precise conditions are required.

The investigation of the directional affects of exposure to X-rays was expanded to include comparative survival following a homogeneous, simultaneous, dorsal-ventral (dual) exposure and both dorsal and ventral exposures in 25R increments between 675R and 800R. The acute dual exposure of 675R at a rate of 126R/min. resulted in a significantly reduced survival compared to a dorsal exposure at a rate of 63R/min. However, the homogeneous dual exposure at a rate of 63R/min. produced a survival identical with that of the dorsal exposure at the same exposure rate over the same exposure time. Therefore, the difference between the acute dual and dorsal exposures was an exposure-rate effect. While a total exposure time of less than 15 min. would not allow time for cell proliferation, the increased exposure time of 5'22" required to exposure animals at a rate of 63R/min. allowed sufficient time for repair of cellular damage since exposure rate effects have been attributed to cell proliferation and/or cellular repair. Differences in the shapes of survival curves for dorsal and ventral exposures suggested that 2 types of damage were involved in the directional effect. Lethal damage, so severe that there was little evidence of repair, was indicated by deaths during the 2nd week post irradiation and was the primary cause of the reduced survival following acute dual exposures. Deaths during the 3rd and 4th weeks appeared to result from sublethal damage that allowed for damaged cells to have a limited amount of proliferation, i.e., the damage was not immediately lethal but could not sustain life indefinitely. It was this later type of damage that benefited by the increased exposure time when the exposure rate was reduced to 63R/min. To override the exposure-rate effect required an increase in dorsal exposure time of 24" longer (25R increase) than the dual exposure, however and additional 48" was required to produce equivalent lethal damage, i.e., at 700R there was no longer a significant difference between dual and dorsal exposures, however, a dorsal exposure of 775R was required to duplicate the survival curve of the acute dual exposure to 750R. In contrast, the ventral exposure to 675R at the same exposure rate as the dorsal exposure demonstrate the importance of the 2 types of cellular damage. Although only 1 mouse died during the 2nd week following ventral

exposure there was a higher mortality during the 3rd and 4th weeks past irradiation than occurred during the entire 30 days following dorsal exposure. To account for this increased mortality, apparently a result of the limited capacity for vital cells to proliferate, it was postulated that there was incomplete repair of more severely damaged cells during ventral exposure which did not occur during dorsal exposure. The exposure differential for dorsal and ventral exposures differed from that of dorsal and dual exposures in that a dorsal exposure of 725R produced a survival curve similar (but not identical) to that of a ventral exposure of 800R. To significantly increase the lethal damage of a ventral exposure required an exposure of 750R. The explanation of this phenomenon rests with the distribution of the target cells in the body. Using a mid-line focus the exposure-rate effect was the same for both dorsal and ventral exposures. In both cases, the circulating cells received the same exposure, however, hematopoietic cells of the spine were proximal to the radiation source during dorsal exposure but distal to it during ventral exposure. In addition, part of the gastrointestinal tract was closer to the radiation source during ventral exposure than during dorsal exposure. This would account for the observation that ventral exposures appeared to produce more irreversible gut damage than dorsal exposures. An exposure reduction by absorption of primary radiation before reaching the critical target was postulated. The hematopoietic cells of the spine received sublethal damage allowing for the differential repair observed, i.e., ventral exposures were affected by a synergestic effect of the exposure-rate plus the lack of homogeneity of the radiation. This was consistent with the fact that ventral exposures to 800R resulted in a 27% 30 day survival and approximately half of the mortality was attributable to damage that limited cell proliferation while 800R was lethal to all mice receiving dorsal exposures. This interpretation of the data was also consistent with the assumption that homogeneity of exposure affected the severity of cellular damage while exposure time affected the amount of repair. However, repair was apparently inititated only during exposure for if it were a continuous process the observed correlation between exposure time and repair would not have existed.

These data clearly reveal the fallacies inherent in converting total-body exposures from roentgens as a calibration in air to an absorbed dose expressed in rads or Grays. Two conversion figures have been used for mice. The coefficient of absorption of 0.93 rads/R has been used on the assumption that the body composition was primarily water. Alternatively a coefficient of 1.02 rads/R assumes that the body composition was a solution of chemical elements in different proportions. This conversion practice was adopted over 30 years ago and physicists have been reluctant to abandon the practice. While physics and chemistry are predictable or "exact" sciences, i.e., given the conditions of an experiment the results may be predicted, biological systems are complex interactions which may be sensitive and reproducible but not predictable, i.e., results must await completion of the experiment and each component of the system may alter the results.

Although directional effects of gamma radiation from Cs-137 has been observed, the interpretation of the data may differ because of the greater penetration and less absorption attributed to gamma rays. Our data envolving gamma radiation appears to be more variable than that obtained with X-rays. This may be

a function of the exposures selected for the tests or a greater influence of the exposure rate. These factors are still under investigation and represent an important aspect of this investigation since radiations of higher energies are used in clinics.

The fact that sequential alternate exposures were not equivalent to the dual exposure was attributed to the exposure-rate effect. A dorsal + ventral exposure was less effective than the dorsal exposure because half of the exposure was subject to the absorption efects of the ventral exposure. Likewise the ventral + dorsal exposures were more effective than the ventral exposure since half the exposure was not subject to the absorption effects of the ventral exposure. However, sequential alternate exposures over the same elapsed time were not equivalent to each other. If repair were initiated only during exposure and limited by the exposure time, survival might be expected to follow the pattern of repair of the direction of the first exposure in the sequence. While this appears to be the type of pattern being established, there is still insufficient data available to evaluate this hypothesis. However, this explanation most closely fits the observed results and no other plausible explanation has become evident.

The radioprotective effect of Nembutal(R) anesthesia was reevaluated as a result of the potential for differential repair demonstrated using ventral exposures of normal mice just described. When C57BL/KaUp mice receiving a ventral exposure within 15 min. of being anesthetized, there was a marked reduction in lethal damage while "sublethal" damage persisted. The overall effect was improved survival compared with normal controls. At 30 min. there was the same amount of lethal damage but improved survival results from the absence of "sublethal" damage. At 60 min. there was less lethal damage and a resumption of some "sublethal" damage. By 90 min. the lethal + sublethal damage decreased the survival to the approximate level of the controls. At 120 min. the lethal damage was again reduced to the level of that seen at 30 and 60 min. while the "sublethal" damage approached that seen at 15 min. This clearly represented a rebound effect. Any hypothesis formulated to explain the mechanism for the radioprotective effects of Nembutal must also include an explanation for the rebound effect and the fact that radioprotection was independent of the degree of sedation.

Significance to Biomedical Research the Program of the Institute:

Experimental marrow transplantation has been demonstrated to be a particularly sensitive system that detected not only the quality of the radiation but directional effects and exposure-rate effects produced by protraction or fractionation of the exposure all of which have not previously been recognized as critical for successful engraftment. Although there has been no standardization of the preparation of the recipients for marrow grafts, the major emphasis has been on depleting donor marrow of subpopulations of cells thought to be responsible for the graft vs. host reaction. An exposure-rate effect has been demonstrated to reduce the effectiveness of the radiation of normal hematopoietic cells in the mouse and for human neoplastic hematopoietic cells in tissue culture (Rhee et al., Rad. Res. 101:519, 1985), how much of the recurrence of neoplasms following marrow transplantation should be attributed to an

exposure-rate effect inherent in the method of preparing the recipient for the graft? While the in vitro data obtained using tissue cultures supports the in vivo data, it cannot be used to evaluate other phenomena involving exposure of circulating and fixed hematopoietic tissues, e.g., the exposure reducing effects of the direction of the 1st exposure of sequential alternate exposure methods as compared with the dual exposure must involve intact animals. Although biological systems would be expected to respond similarly to general effects of ionizing irradiation, it is necessary to reproduce these experiments using radiation of greater energy e.g., gamma rays from Cs 137.

The prolonged radioprotective effects of anesthesia would have a profound effect on the reproducibility of all experiments involving preirradiation operative procedures as well as irradiation under sedation. The significance of these experiments will increase as more in vivo experimentation will be required to be performed under sedation as enforcement of the stricter animal care guide lines increases.

Proposed Course of the Project:

First priority will be to continue the effort to publish existing data and conduct experiments considered essential by reviewer. There will be a continued effort to define, more specifically, the quality of X-rays and the subtle changes that alter the biological data. Experiments comparable with those conducted using X-rays will continue using gamma radiation from Cs-137.

Recent clinical reports that T-cell depletion to avoid a GVH reaction following marrow transplantation results in a high incidence of both treatment failure and recurrent leukemia requires a reevaluation of this procedure. These results are reminiscent of some old basic experiments in which certain types of marrow pretreatment interfered with engraftment. If a parallel cause and effect relationship exists, it might be possible to develop an experimental system that would resolve this problem.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08706-15 OD

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Alteration in Gene Expression During Mammary Gland Tumorigenesis

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

PI: Gilbert H. Smith Research Biologist OD DCBD NCI

COOPERATING UNITS *(if any)*

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

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☐ (a1) Minors

☐ (a2) Interviews

B

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

I have been studying alterations in expression of MMTV proviral and differentiation-specific genes such as casein and α-lactalbumin at different points in mammary gland tumorigenesis in a "clean" inbred mouse strain (C3H/Sm). This strain is of interest because "normal" expression of MMTV RNA transcripts in mammary gland is not accompanied by the appearance of viral structural proteins or by virions. In addition, there is no detectable rearrangement or amplification of MMTV proviral DNA wihin the cellular genome of mammary tumors developing in these mice. Nevertheless mammary tumorigenesis, either experimentally induced or spontaneous, results in a significant increase in the abundance of MMTV gene transcripts, especially a 2.2 Kb RNA containing only MMTV long terminal repeat (LTR) sequences. This transformation-related increase in abundance of MMTV LTR RNA has been extended to include preneoplastic mammary lesions (hyperplastic alveolar nodules) in C3H/Sm mice as well. Outgrowth lines of these preneoplastic lesions possess enhanced activities of their casein and α-lactalbumin genes in the absence of hormonal stimulation and release humoral factors which profoundly affect the development and differentiation of normal mammary tissue in mammary fat pads distal to their location in the host. We have tentatively identified several proteins encoded by MMTV LTR RNA which are present in these tumors and are abundantly represented in purified preparations of MMTV preprocapsids (intracytoplasmic A particles). Some of these proteins bind strongly to nucleic acids and may have the ability to unwind native double-stranded DNA. Competition studies indicate that these LTR ORF proteins show definite specificity for binding to LTR DNA sequences. Studies are in progress to determine whether these proteins have trans-acting potential for gene expression.

GPO 914-918

Major Findings:

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

In our experiments, a 2.2 Kb MMTV LTR-containing RNA is increased in abundance in chemically, hormonally, or spontaneously transformed mammary epithelium, both preneoplastic and neoplastic. The enrichment of this specific proviral transcript occurs in the absence of MMTV structural protein synthesis and without acquisition of new viral DNA by the transformed cell genome. This increase is especially pronounced in mammary adenocarcinomas originating from ductal hyperplasias. However examination of RNA isolated from serially-transplantable mammary preneoplastic outgrowth lines which exhibited ductal hyperplasia showed no difference in abundance of LTR ORF RNA as compared to normal glands within the same host. Attempts to increase the levels of MMTV RNA in preneoplastic outgrowths *in vivo* with fat pad implants of dexamethosone/cholesterol pellets or pituitary isograft implantation under the kidney capsule were not successful. In agreement with this result, no difference in the incidence of mammary tumor development was observed among dexamethasone-treated, pituitary isograft-stimulated, or control outgrowths.

With polyclonal antibodies raised against synthetic peptides derived from the nucleotide sequences of the MMTV LTR open reading frame (ORF) (kindly provided by Dr. R. D. Cardiff, University of California, Davis), we have probed mammary tumor extracts and proteins synthesized in cell-free translation systems by polyadenylated tumor RNA for the translation products of our 2.2 Kb transformation-sensitive MMTV LTR RNA transcript. We have identified several LTR ORF-related proteins tightly associated with MMTV preprocapsids purified from MMTV-infected tumors. Some of these LTR ORF-related proteins bind strongly to DNA. In competition DNA binding experiments, some of these LTR-related proteins demonstrated substantial preference for binding to MMTV LTR DNA suggesting that they may play a regulatory role in MMTV gene expression or alternatively function to maintain free ends in MMTV DNA molecules thereby aiding recombination between viral and host DNA. These observations provide a basis for clarification of the role of the LTR ORF in MMTV replication and tumorigenesis. Until now, the MMTV LTR ORF protein, although predicted from the nucleotide sequence of the LTR open reading frame, had not been identified *in vivo*.

Cell-free translation of polyadenylated cellular RNAs from C3H/Sm tumors containing highly levels of the MMTV LTR ORF 2.2 Kb RNA produced low molecular weight proteins which were immunoprecipitable by antiserum directed against LTR-ORF-encoded synthetic peptides, but no immunoprecipitable MMTV virion-related structural proteins could be detected with antisera directed against whole MMTV, gp52 *env* or p27 *gag* in the same reaction mixture. Further study

showed this to be true even when purified translatable MMTV virion RNA (35S) was added to the incubation reaction or when poly A^+ RNAs from MMTV-infected C34/Sm tumors were translated *in vitro*. We are currently testing the role of the endogenous C3H/Sm LTR RNA transcripts in this post-transcriptional regulation of MMTV RNA expression.

In experiments designed to determine the requirement of basement membrane deposition for functional differentiation of virgin mouse mammary epithelium, it was discovered that hormone-induced lactational response and DNA synthesis were absent in mammary explants deprived of proline (a non-essential amino acid) but not those deprived of glycine. In related experiments it was found that collagenase-separated mammary epithelial cells placed in tissue culture also required proline for growth but only when plated on type I collagen, the presence of type IV collagen in the culture substratum removed the proline requirement. Studies to elucidate the mechanism of this auxotrophy *in vivo* and its relationship to growth regulation are planned.

Publications:

Vaage, J., Smith, G. H., Asch, B. B., and Teramoto, Y.: Mammary turmorigenesis and tumor morphology in four C3H sublines with or without exogenous mammary tumor virus. Cancer Research 46: 2096-2100, 1986.

Smith, G. H., Doherty, P. J., Stead, R. B., Gorman, C. M., Graham, D. E., and Howard, B. H.: Detection of transcription and translation *in situ* with biotinylated molecular probes in cells transfected with recombinant DNA plasmids. Analytical Biochemistry 156: in press, 1986.

Smith, G. H., Young, L. J. T., Benjamini, E., Medina, D., and Cardiff, R. D.: Proteins antigenically related to peptides encoded by the mouse mammary tumor virus long terminal repeat are associated with intracytoplasmic A particles. J. Gen. Virology, in press, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08901-2 OD

PERIOD COVERED

October 1, 1985 to September 30, 1986

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

Studies of Animal Cell Adhesion

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

PI:	Samuel W. Luborsky	Chemist	OD DCBD NCI
Other:	Kenneth M. Yamada	Chief, Membrane Biochemistry Section	LMB DCBD NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Proteins such as fibronectin, whose function is to facilitate cell adhesion to extracellular substrate surfaces or to other appropriate cells, are synthesized by cells of most vertebrate species. Assays are available to quantitate these proteins by measuring the percentage of cells which attach and spread on the plastic surface of a tissue culture well. To study the mechanism of cell adhesion Yamada has obtained certain fibronectin-derived peptides and has ranked them in order of their effect upon fibronectin mediated BHK cell adhesion. To investigate what structural feature(s) the active peptides have in common, I have continued work with molecular models to construct some of these peptides, using literature values of bond lengths and angles. Although it was at first felt that essentially complete geometric parameters were available, it soon became apparent that enough variability in conformation still existed to preclude any conclusions concerning important structural features involved in cell binding and adhesion. Another approach to this problem was to determine the percentage of aggregation at any concentration for each peptide, in order to fit the activity level to concentration of active species. The degree of aggregation was measured by sedimentation equilibrium experiments in the analytical ultracentrifuge. A number of such measurements were made and percent dimer determined for each peptide. These data will be included in any models of the active peptides. Such models should help us to determine the crucial structural features involved in cell binding and adhesion.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

Cells of most vertebrate species are anchorage dependent and therefore undergo mitosis and proliferate only when firmly attached to a surface. These cells contain proteins whose function is to facilitate cell adhesion to extracellular substrate surfaces, or to other appropriate cells to form tissues and organs. Assays have been developed to detect and quantitate these proteins, using cell spreading on a substratum as a measure of cell adhesion. Fibronectin is one of these adhesion proteins which provides an essential physiological bond between substratum and anchorage-dependent cell. As one approach to the study of the mechanism of cell adhesion, Yamada has obtained and purified certain fibronectin-derived peptides, and is studying their effect upon fibronectin-mediated BHK cell adhesion to the surface of the plastic tissue culture wells. From assays such as these, Yamada has ranked the various peptides in order of their relative activities in promoting cell adhesion to the fibronectin and other adhesion proteins. Clearly, an immediate question is what structural feature(s) the active peptides have in common, which might be different in the inactive peptides. I have, therefore, continued work with molecular models to construct some of these peptides. Data for most of the bond lengths and bond angles were found in the literature. Although at first it was felt that essentially complete geometric parameters were available, it soon became apparent as construction proceeded, that enough variability in conformation still existed to preclude any new, useful generalizations/conclusions being made concerning structural features which might be involved in cell binding and adhesion.

Another approach to this problem was suggested by the fact that although the activity of most of the peptides could be explained by a simple model, some peptides could not be fitted to this same simple model. We wondered if the state of aggregation of these peptides might influence their activities. The degree of aggregation could be easily measured by sedimentation equilibrium experiments, in which under suitable conditions we determined that equilibrium was attained in an overnight run. Sedimentation equilibrium experiments were therefore carried out on a number of these peptides and the percent dimer of each peptide was determined at a series of peptide concentrations. These data will be included in any models of the active peptides. Such models should help us to determine the crucial structural features involved in cell binding and adhesion.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08902-2 OD

PERIOD COVERED
October 1, 1985 to September 30, 1986

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Chemical Characterization of an Immunosuppressive Glycoprotein

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

PI:	Sidney Shifrin	Chemist	OD DCBD
Other:	Andrew V. Muchmore	Medical Officer	MET DCBD

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	2.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

Succinylation of lysyl residues of uromodulin results in the unfolding of the secondary structure of the glycoprotein but does not interfere with the ability of the urinary glycoprotein to suppress the immune response. Trinitrophenylation of lysyl residue does not affect the structure nor does it affect the immunosuppressive properties. Modification of tyrosyl residues by iodination, nitration or O-acetylation does not affect the ability of uromodulin to suppress lymphocyte proliferation. In fact, it appears that the protein moiety of uromodulin plays a minor role in suppressing the immune response. The activity appears to reside in the oligosaccharide moiety.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

Uromodulin was isolated from human pregnancy urine and was purified so that more than 95% of the protein had a molecular weight of 85,000. The remaining 5% of the protein had a molecular weight of 30,000. Uromodulin was found to consist of 20-30% carbohydrate as determined with orcinol. The following amino acid residues were modified in an attempt to determine their role in the immunosuppressive properties of uromodulin: lysine, tyrosine and cystine. Lysyl residues were modified by reaction with trinitrobenzenesulfonic acid and with succinic anhydride. Tyrosyl residues were modified by reaction with iodine, N-acetylimidazole, and tetranitromethane. The cystine residue was modified by reduction with dithiothreitol followed by reaction with iodoacetic acid.

Although the secondary, tertiary, and quaternary structure of uromodulin was modified as determined using physicochemical methods and migration in polyacrylamide, the immunosuppressive properties were not altered. In fact, the protein was extensively digested with pronase without affecting the immunosuppressive properties. The oligosaccharides of uromodulin were isolated and were found to be immunosuppressive. Further studies on the structure of the oligosaccharide should be carried out to determine the role of the individual sugars in the immunosuppressive properties of uromodulin.

Publications:

None

CONTRACT RESEARCH SUMMARY

Title: Maintain an Animal Holding Facility and Provide Attendant Research Services

Principal Investigator: Ms. Leanne DeNenno
Performing Organization: Bioqual, Inc.
City and State: Rockville, MD

Contract Number: NO1-CB-33876
Starting Date: 1/11/83 Expiration Date: 10/31/87

Goal: Maintain colonies of inbred mice (12,000 animals), inbred rats (500 animals), and rabbits (40 animals) and carry out selected breeding protocols with these animals as specified by the project officer. These animals are to be maintained in support of intramural research programs in the Immunology Branch, NCI.

Approach: Colonies of mice, rats, and rabbits are to be housed and fed according to National Research Council standards. Technical manipulations and breeding are to be carried out as directed by the project officer.

Progress: 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 Officer: Dr. David H. Sachs
Program: Immunology Support
Technical Review Group: Intramural Support Contract Subcommittee A
FY 86 Funds: $460,775

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 6000 mice were received into the contract facility during the first year, and the facility contained between 2500 and 2800 mice at any given time. Two preparations each of cytomegalovirus and influenza virus were made. Approximately 1200 radiation chimeras, 3600 i.p. inoculations, 2500 i.v. injections, 1500 tail bleeds, 50 thymus grafts, 100 virus plaque assays, 160 skin grafts, 50 embryo transfers, 130 lymphocyte preparations, and 200 ELISA tests for cytomegalovirus antibodies were performed during FY 1986. The mice have been delivered to the Immunology Branch laboratories as requested, and accurate 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 Support
Technical Review Group: Intramural Support Contract Subcommittee A
FY 86 Funds: $313,029

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: Analysis of cell surface antigens is performed by two different detection systems: serology and cellular typing. The serologic analysis is performed using carefully screened alloantisera in assays of complement-dependent cytotoxicity. The cellular analysis is done by PLT assay which involves analyzing secondary restimulation of lymphocyte populations selectively immunized against alloantigens in mixed lymphocyte culture.

Progress: During the tenure of the contract, serotyping has been performed on 230 cell samples. The quality of the typing was consistently excellent. However, two factors have prompted an elimination of serotyping from the contract: first, serious cutbacks in funding; and second, major improvements in the on-site serotyping capabilities at NIH by Dr. R. Davies and T. Simonis. Consequently, future serotyping will be performed at NIH.

The remaining critical element in the contract relates to cellular typing, particularly for the DP antigens. Disruption of progress in this area of typing accompanied the move of a previous principal investigator. However, this typing has resumed under the very able direction of Dr. David Eckels. The contractor has generated, expanded and frozen DP-specific PLT reagents. Progress is currently being made in provision for typing for DPw6. Furthermore, they have characterized an extensive panel (146 total) of local cells for DP antigens. These reagents and the typing assay are being carefully validated and used for DP typing of experimental and control samples.

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 officer: Dr. J. Stephen Shaw
Program: Intramural Research Support
Technical Review Group: Ad Hoc Intramural Technical Review Group
FY 86 Funds: $58,489.00

A

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-CB2-5584
Starting Date: 02-22-82 Expiration Date: 1-31-87

Goal: Induction, transplantation, preservation and shipping of plasmacytomas, T- and B-cell lymphomas in mice. Breeding of (congenic) strains of mice to find genes controlling susceptibility and resistance to the induction of plasma cell tumors by pristane; maintenance of wild mouse colony.

Approach: Maintain a closed conventional colony of inbred and congenic strains of mice, suitable for maintaining mice for long term plasmacytoma induction experiments. Develop BALB/c congenic strains carrying plasmacytomagenesis resistance (PCT-R) genes. Carry out procedures for identifying markers used in the construction of congenic strains. Maintain colonies of pedigreed wild mice. Harvesting and shipment of N_2-frozen transplantable tumors, serum, ascites, tissues, high molecular weight DNA, pedigreed breeders to qualified investigators and collaborators.

Progress: The contractor has continued to supply the Laboratory of Genetics with essential biological materials for ongoing research projects. This year we have completed a comprehensive histological study of oil granuloma formation plasmacytoma development in BALB/cAn mice and compared these changes with those in four plasmacytoma resistant strains. We are continuing to develop BALB/c•DBA/2 congenics that carry resistance genes and the testing of the CXS recombinant inbred mice. Contractor continues to supply tumor tissue DNA from pedigreed mice in the inbred and wild colonies for ongoing studies in the Laboratory of Genetics. Contractor has assisted in carrying out experiments on the co-plasmagenic activity of transforming viruses containing myc and ras oncogenes, as well as supplying the tumors for analysis. The contract continues to be an actively used source of biological materials.

Significance to Cancer Research: Provides essential support for the study of plasmacytomagenesis (carcinogenesis) with the specific goal of determining the genetic basis of susceptibility to tumor induction by mineral oil. Supplies essential biological material for investigators studying the biology of neoplastic plasma cells, tumor immunology, the genetics of immunoglobulins, and immunoglobulin synthesis.

Project Officer: Dr. Michael Potter
Program: Immunology Support
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 86 Funds: $791,608.00
B

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-44020
Starting Date: 6/30/84 Expiration Date: 6/29/87

Goal: To perform radioimmunoassays of immunoglobulin molecules as well as ELISA assays of soluble interleukin-2 receptor antibody molecules in lymphocyte culture supernatants or in biological fluids.

Approach: The contractor is to quantitate human IgG, IgA, IgM, IgE, lambda and kappa light chains, and mouse IgG in various fluids using double antibody radioimmunoassay procedures and reagents defined and supplied by the project officer. Furthermore, the contractor is to measure antibodies produced by lymphocytes stimulated by antigens in vitro. This contract provides critically required research support for the study of immunodeficiencies that are associated with a high incidence of malignant transformation and with human B- and T-cell leukemias.

Progress: The contractor has established radioimmunoassays for IgG, IgA, IgM, and lambda and kappa light chains of man and IgG of mice. These assays were used to quantitate immunoglobulin and antibody synthesis by human lymphocytes in in vitro cultures. Patients with the adult T-cell-leukemia-associated human T-cell leukemia/lymphoma virus were shown to have a malignant expansion of suppressor T cells that react with a monoclonal antibody anti-Tac that identifies the inducible receptor for T-cell growth factor, whereas patients with the Sezary syndrome have a malignant expansion of helper T cells. The assays of mouse IgG in human plasma were used in studies of the metabolism of anti-Tac in the therapy of adult T-cell leukemia. The contractor has established ELISA assays for the soluble 45 kd form of the IL-2 receptor binding peptide. The level of this peptide that appears in the serum and urine parallels the activity of T-cells in the body. The assay has been of value in following the efficacy of therapy of hairy cell leukemia and adult T-cell leukemia. An elevated level of the serum IL-2R was shown to be the best prognostic indicator in non Hodgkin's lymphoma. Elevated marker levels have been demonstrated prior to organ allograft rejection. These studies are defining the nature of disorders of the immune system related to cancer.

Significance to Cancer Research: These studies helped elucidate the abnormalities of the immune system associated with the development of cancer. They have assisted in the categorization of malignancies of the lymphoid system and in defining lymphokines involved in suppression of humoral immunity. They are required for therapeutic protocols involving the use of the anti-Tac monoclonal antibody. 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: Intramural Support Contract Subcommittee A
FY: '86 Funds: $105,220 est.

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-25585
Starting Date: 3/1/82 Expiration Date: 1/31/87

Goal: To maintain a colony of inbred pedigreed strains of mice which are needed to support ongoing NCI intramural research in transplantation immunology.

Approach: The contractor maintains a colony of approximately 40 special inbred and congenic resistant strains of mice by pedigreed brother-sister mating. Quality control testing is carried out at each generation by cytotoxicity typing of breeders from each strain. Alloantisera are raised between mouse strains to assist in this quality control typing, and sera and animals are shipped by the contractor to collaborating investigators at NIH and elsewhere.

Progress: The contractor has maintained all inbred and congenic resistant strains of mice in excellent condition. Breeding of each strain and of hybrid strains, recordkeeping, and quality control testing have all been highly satisfactory. A backcrossing program has been instituted for all congenic resistant strains in order to keep the backgrounds of these strains identical. This involves backcrossing of each congenic to the reference background line once every 6-10 generations. This program has also been very satisfactory to date. 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 are now being done by the contractor, with the purpose of developing monoclonal antibodies to histocompatibility antigens. Antisera for histocompatibility antigen typing have been prepared in various combinations and have been found to be excellent reagents. A series of new strain-restricted typing sera have been produced in order to distinguish each strain in the colony from all other strains. Shipping of animals and sera to collaborating investigators at NIH and elsewhere has been satisfactory. The animals shipped have generally been of excellent health and have provided breeding stock for the production of experimental animals in numerous laboratories. Computerization of records and reports has been performed and has led to efficient data handling. Hybridoma reagents have been produced, stored and shipped starting with cell lines developed by the Project Officer.

Significance to Cancer Research: This animal facility is needed for the breeding and maintenance of these inbred congenic resistant strains of mice. These animals make possible research on individual histocompatibility antigens and, in particular, the role of the major histocompatibility complex in the transplantation of tissues and cells and in the immune response to cancer.

Project Officer: Dr. David H. Sachs
Program: Immunology Support
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 86 Funds: $647,396 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: N01-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 carcinogenes in various feral strains of Mus musculus and other species of Mus. Breeding of (cogenic) strains of feral Mus musculus which 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-germ line negative M. musculus CZII strain. Harvesting and shipment of N_2-frozen primary and transplanted mammary tumors, tissues, and pedigreed feral mice to qualified investigators and collaborators.

Progress: The contractor has continued to maintain all of the colonies of feral mice in excellent condition as well as satisfactorily perform the breeding program, quality control, and maintenance of records. The int-2 common insertion site for MMTV has been shown by genetic crosses to be located 16cM from the Hbb locus on chromosome 7. The gene order is H-ras-1-Hbb-int-2. The MMTV (CzechII) virus has been transmitted to the BALB/c mouse strain. A new strain which contains only the MTV-2 endogenous MMTV genome on the CzechII genetic background is near ready for inbreeding. The MTV-2 locus is associated with a high incidence of pregnancy dependent mammary tumors.

Significance to Cancer Research: Provides essential support for the study of mammary tumorigenesis with the specific goal of dissecting the genetic and molecular interaction between genetically transmitted MMTV genomes and exogenous carcinogens. Provides essential biological material for other investigators studying the biology of mouse mammary tumor virus as well as other classes of genetically transmitted retroviral genomes.

Project Officer: Dr. Robert Callahan
Program: Immunology Resource
Technical Review Group: DEA; Ad Hoch Intramural Technical Review Group

FY 86 Funds: $109,500

CONTRACT RESEARCH SUMMARY

Title: Hybridoma Assays and Related Laboratory Tests

Principal Investigator: Dr. Ronald Gillette

Performing Organization: Meloy Laboratories, Inc.
City and State: Springfield, Virginia

Contract Number: NO1-CB-33872
Starting Date: 10-01-82 Expiration Date: 12-30-86

Goal: The contractor maintains a hybridoma production laboratory in which selected hybridomas are cloned and screened for specific monoclonal antibody production. Tissue sections are cut from paraffin embedded and fresh surgical specimens supplied by the project officer; these sections are stained using immunoperoxidase techniques.

Progress: The contractor produced mass qualities of tissue culture supernatants and ascites fluids containing monoclonal antibodies of interest. Extracts of human tumor tissue and human tumor cell lines were prepared for use in characterizing assays for new and existing antibodies. Antigen modulation studies were also performed using human tumor cell lines and biological response modifiers to maximize antibody recognition of target antigens.

Significance to Cancer Research: This contract has been used to process the large quantity of tissue sections and perform radioimmunoassays needed to screen monoclonal antibodies for specificity. This contract was also needed to produce large quantities of cell culture supernatant fluids, ascites fluids, and testing for monoclonal antibody research in anticipation of clinical trials and to supply the numerous laboratories requesting these reagents.

Project Officer: Dr. Jeffrey Schlom
Program: Immunology Resource
Technical Review Group: DEA; Intramural Support Contract Proposal Review Committee
FY 86 Funds: $0

CONTRACT RESEARCH SUMMARY

Title: Construction and Characterization of Genomic DNA Libraries

Principal Investigator: Dr. J. Norman Hansen

Performing Organization: University of Maryland
City and State: College Park, MD

Contract Number: NO1-CB-3-3934
Starting Date: 08-01-83 Expiration Date: 07-31-86

Goal: Construction of recombinant bacteriophage (libraries) containing entire genomic representation of 5-10 different mouse species and sub-species. Screening of these libraries with a variety of immunoglobulin variable and constant region gene probes for selection of corresponding homologues. Characterization of the isolated clones containing the genes of interest.

Approach: Genomic DNA from species and sub-species selected will be introduced into appropriate bacteriophage following partial digestion with restriction endonuclease enzymes. These libraries will be assayed by filter hybridization to select genes homologous to immunoglobulin genes from inbred stains. Selected genes will then be characterized by restriction enzyme digestion (mapping), hybridization with radioactively labeled DNA probes, and then introduced into plasmid vectors. Preparative amounts of subclones will then be supplied to P.O. for further analysis or sequenced at the contract site as directed.

Progress: Recombinant bacteriophage DNA libraries have been prepared from five different wild mouse species. All libraries have been screened for genes homologous to immunoglobulin C_K, V_K and V_H sequences and corresponding positive clones have been isolated in each case. To date, a V_H gene family has been sequenced from three species. The C_K gene, as well as the 5' kappa chain enhancer region, has now been cloned and sequenced from all five libraries providing a model for evolution of a single copy gene as well as an enhancer sequence. Members of a selected V_K family have also been isolated from all species and nucleotide sequence determination, which will occupy the remainder of the contract performance period, is in progress.

Significance to Cancer Research: One of the possible mechanisms involved in the generation of neoplasia is mutations occurring in structural genes. The present contract supplies materials to permit an assessment of mutational events occurring in multi-gene families in the germline. This analysis will provide information on the occurrence of such diverse events as point mutation, recombination and gene interaction.

Project Officer: Dr. Stuart Rudikoff
Program: Intramural Research Support
Technical Review Group: Ad Hoc Intramural Support Contract Review Group
FY 86 Funds: 0

B

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