











**Intramural Activities**

October 1, 1988 - September 30, 1989

# 89 annual report

Division Of

# Cancer Biology and Diagnosis

U.S. DEPARTMENT OF HEALTH AND  
HUMAN SERVICES  
National Institutes of Health

NATIONAL  
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U.S. DEPARTMENT OF HEALTH AND  
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NATIONAL CANCER INSTITUTE

ANNUAL REPORT

October 1, 1988 through September 30, 1989

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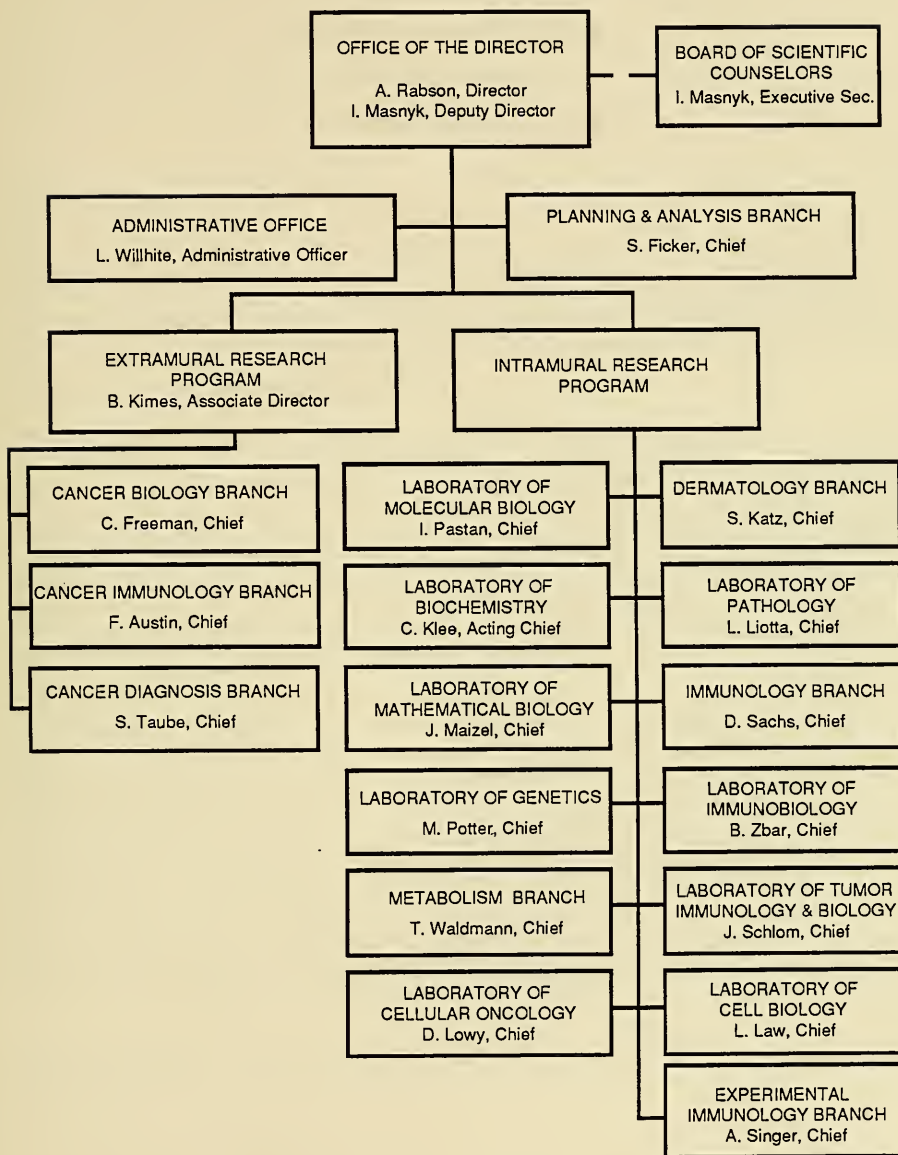
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**DIVISION OF CANCER BIOLOGY AND DIAGNOSIS  
NATIONAL CANCER INSTITUTE**





DIVISION OF CANCER BIOLOGY AND DIAGNOSIS  
NATIONAL CANCER INSTITUTE

INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1988 through September 30, 1989

INTRODUCTION

The mission of the Division of Cancer Biology and Diagnosis is to support laboratory and clinical investigations in cancer biology, immunology, and diagnosis. The division consists of an extramural research program and an intramural research program. The Extramural Research Program administers a large research program comprised primarily of investigator-initiated grants. Three major areas of cancer research are addressed: tumor biology, immunology, and cancer diagnosis.

The Intramural Research Program consists of thirteen independent laboratories and branches on the NIH Bethesda campus and at the Frederick Cancer Research Facility. They are the Laboratory of Genetics, the Laboratory of Molecular Biology, the Laboratory of Biochemistry, the Laboratory of Cellular Oncology, the Laboratory of Pathology, the Dermatology Branch, the Metabolism Branch, the Immunology Branch, the Experimental Immunology Branch, the Laboratory of Cell Biology, the Laboratory of Tumor Immunology and Biology, the Laboratory of Immunobiology, and the Laboratory of Mathematical Biology. Each of these laboratories is headed by a scientist of international stature, three of whom are members of the National Academy of Science.

The research conducted in the intramural program covers a broad range of investigations from gene regulation in prokaryotes to clinical studies of human cancer. A common goal of many of these studies is to determine the molecular mechanisms involved in the regulation of gene expression, with particular emphasis on the mechanisms responsible for the aberrant properties of a cancer cell. There is a broad based program in immunology which investigates the fundamental questions involved in the regulation of the immune response, and the derangements that allow tumor cells to escape immune surveillance. The diversity and high quality of these studies reflects the varied interests and expertise of the scientists in each laboratory. Interactions among scientists are encouraged, and the exchange of information and technical expertise has led to the development of entirely new ways of approaching the some of the most puzzling problems of human cancer.

The following summary describes the research activities and major accomplishments in each of the DCBD intramural research laboratories during the past year.

## LABORATORY OF GENETICS

The major focus of the work in the Laboratory of Genetics, directed by Dr. Michael Potter, is on B cell tumor development in mice, with particular emphasis on the plasmacytoma system. The BALB/c mouse is uniquely susceptible to plasmacytoma development following the intraperitoneal injection of pristane or other paraffin oils. Dr. Potter's group is attempting to identify genes that determine resistance (R) and susceptibility (S). Two genes have been tentatively identified through linkage association: Pctr-1 (chr 4) and Pctr-3 (chr 17). A congenic strain BALB/c.D2-Fv-1, that carries Pctr-1, has been developed. After 19 backcrosses this mouse develops less than 20% plasmacytomas. Dr. Potter's group is attempting to localize Pctr-1 using a 3-factor cross. Much effort is directed to finding a closely linked gene.

Dr. Beverly Mock has continued to study a plasmacytoma resistance gene that controls plasmacytoma development induced by a combination of pristane and RIM virus (the virus that contains a myc oncogene and v-Ha-ras). She has found that the BALB/c.D2-Dag mouse, which is as resistant as the DBA-2 parent to RIM/pristane induction, carries an additional DBA/2 gene, MMTV-13, that is not linked to Ly-6 (chr 15). A C.D2 Ly-6 congenic is susceptible to both pristane and pristane/RIM induced plasmacytomagenesis. She has screened 25 markers for linkage to this R/S gene. Her laboratory has developed libraries of mouse chrs 4 and 15 and is developing new markers on chromosomes 4 and 15 for this purpose. Since BALB/c.D2-Dag is a congenic mouse, this is an important new direction.

Other studies focus on the role of the raf-1 oncogene in plasmacytomagenesis. The J3 virus, which contains a mutant and defective raf gene and a v-myc gene does not transform plasma cells. However, when this virus is passaged, it acquires plasmacytomagenic activity by correcting the frameshift mutation in v-raf. This finding opens up a new area because this virus undergoes further mutational change in acquiring plasmacytomagenic activity.

There is a continuing interest in identifying genes that cooperate with c-myc in plasmacytomagenesis. It has not been possible to implicate members of the ras gene family. Myc is apparently activated indirectly by the reciprocal translocations of rcpt(6;15). In man the activation of myc is thought to be mediated by base substitution mutations in c-myc. Attempts to determine whether similar mutations occur in the mouse have not progressed, because of the unavailability of the 5 prime UT region of the c-myc gene. This region has now been cloned, and the myc genes from plasmacytoma, ABPC-22, which has no translocation, have been cloned. One allele contains an unidentified insertion. The evidence indicates there are base substitution mutations in myc in the rcpt(6;15) translocations.

### Myeloid Tumors

Dr. Linda Wolff has continued her work on the induction of monoblastic tumors by the injection of Moloney virus in pristane-conditioned mice. She has shown that the virus must be injected intravenously and, further, that these tumors can be prevented by treating mice with indomethacin. Recently she has shown that splenectomy can inhibit myeloid tumor development. Current studies are

directed towards studying the role of prostaglandins in this process. The Moloney virus inserts into the c-myb oncogene in virtually all of the monoblastic tumors. During the past year Dr. Grace Shen-Ong has focused on the characterization of the protein products of the normal c-myb (75 kd) and tumor specific truncated myb (70 kd) in murine cells.

Studies in Dr. Sandy Ruscetti's laboratory have concentrated on determining the mechanisms by which the spleen focus-forming virus abrogates the erythropoietin dependence of erythroid cells and causes erythroleukemia. Her data indicate that the transmembrane portion of the viral envelope glycoprotein is responsible for conferring factor-independence on erythroid cells and that the mechanism does not involve factor production or an increase in the number or affinity of membrane receptors. Considerable progress has been made in understanding the molecular basis for the resistance of certain strains of mice to erythroleukemia induced by Friend MuLV. The data suggest that resistant mice carry a unique provirus that encodes an envelope glycoprotein that blocks the spread of mink cell focus-forming viruses in these mice. Mice carrying this provirus also have a greatly reduced susceptibility to thymomas induced by Moloney MuLV.

### Antibody Protein Interactions

In order to define the structural basis of molecular recognition in protein-protein interactions, Dr. Sandy Smith-Gill's group has analyzed in detail the antibody antigen interface of monoclonal antibodies specific for the defined protein lysozyme, focusing on two antibodies for which the X-ray structures of the Fab:lysozyme complexes have been determined. The effects of specific amino acid residues on specificity have been examined by cloning, expressing, and performing site-directed mutagenesis on the rearranged HyHEL-10 genes, and by site directed mutagenesis of cloned and expressed lysozyme genes. These two mutagenic approaches have produced unexpected results indicating nonadditive and long-range interactions, and are raising provocative questions concerning the nature of specificity and its relation to the biology and regulation of the immune response.

### **LABORATORY OF MOLECULAR BIOLOGY**

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

### Regulation of Gene Activity

The EGF receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To study regulation of the EGFR gene, Dr. Pastan's group has isolated and sequenced the promoter region. They found that it is very GC-rich, but does not contain a TATA sequence. It does contain many transcription factor binding sites including four Sp1 sites and sites for at least three other factors. One of these factors, a 120 kilodalton protein termed ETF, binds to three sets of GC-rich sequences and stimulates EGF receptor transcription in cell-free extracts. The function of this protein, which may be a transcriptional repressor, is under investigation.



## Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells

Resistance to multiple drugs is a major impediment to the successful chemotherapy of human cancers. One mechanism of multidrug resistance (MDR) is the expression of a 170,000 dalton energy-dependent drug efflux pump (P-glycoprotein) which confers resistance to many of the chemotherapeutic agents used to treat cancer. Expression vectors into which the MDRI cDNA is cloned confer the complete MDR phenotype on drug-sensitive cells, as does a retrovirus carrying the MDRI gene. During the past year, Dr. Pastan and his colleagues has constructed a chimeric P-glycoprotein, in which a functional adenosine deaminase (ADA) molecule is attached to the C-terminus. Introduction of this cDNA into cells results in drug resistance and ADA activity simultaneously. Transgenic mice carrying the human MDRI gene expressed in bone marrow have been produced, and shown to be resistant to daunomycin-induced leukopenia. These mice will be useful in the testing of new drugs designed to overcome multidrug resistance. Expression of MDRI RNA and P-glycoprotein normally occurs in kidney, liver, colon, and adrenal, and in cancers derived from these tissues which are intrinsically resistant to chemotherapy. Acquired drug resistance in other cancers is associated with increased MDRI RNA levels.

## Immunotoxin and Oncotoxin Therapy of Cancer Cells

*Pseudomonas* exotoxin (PE) or genetically modified forms of PE have been attached to monoclonal antibodies (MAbs) or growth factors to create cell-specific cytotoxic agents. Native PE attached to MAb OVB3 is cytotoxic to ovarian cancer cells, inhibits the growth of ovarian cancer in mice, and is being currently tested in a Phase I trial in women with ovarian cancer. Mutant PE molecules in which domain I has been replaced by TGF $\alpha$ , IL2, IL4, or IL6 have been created by the technique of gene fusion and the chimeric proteins produced in *E. coli*. TGF $\alpha$ -PE40 kills cancer cells with EGF receptors. IL2-PE40 kills certain types of leukemia cells as well as activated T cells involved in allograft rejection. IL6-PE40 is cytotoxic to some myeloma cells. CD4 has also been fused to PE40 to create CD4-PE40, a chimeric toxin which kills cells infected with HIV. Such molecules may be useful in the elimination of tumor cells or other cells such as HIV infected cells responsible for several human diseases. Studies of the structure-function relationships of *Pseudomonas* exotoxin (PE) are suggesting new ways to construct immunotoxin molecules to increase their rate of endocytosis and thereby enhance their cytotoxic activity.

## Functions, Structure, and Regulation of Receptors for Cell Adhesion Proteins

Receptors for key extracellular proteins such as fibronectin, collagen, and laminin are thought to mediate adherence, migration, and invasion by tumor cells, as well as analogous processes by certain normal cells. Dr. Ken Yamada has been characterizing proteins that bind collagen or fibronectin. One collagen-binding protein is a transformation-sensitive heat-shock protein. A second binding protein termed VLA-2 may be central to normal and tumor cell penetration of collagenous matrices. Fibronectin receptors were shown to be altered in quantity and/or distribution in vivo and in vitro. Levels are increased in Rous sarcoma-virus induced tumors, and its membrane localization is altered in a variety of tumor cells. The structure/function studies of the



human fibronectin receptors have shown that the beta subunit, shared by other receptors, is required for normal cell attachment to fibronectin, laminin, and collagen, while the alpha subunit provides specificity. This fibronectin receptor is required for organization of an extracellular fibronectin matrix, and to restrain migration of normal fibroblasts as part of its function in maintaining the stationary cell phenotype. It thus appears to play a central role in regulating normal cell adhesion and migration.

### Structure and Roles of Transformation-sensitive Cell Surface Glycoproteins

Fibronectin plays a crucial role in cell adhesion, migration, invasion, and metastasis. Four key sequences in fibronectin involved in its recognition by cells are being characterized, and the role of each of these sites is being defined. Vitronectin, another major cell adhesion protein, was found to be regulated by an extracellular molecule termed PG-M, a novel proteoglycan that modulates adhesion via its chondroitin sulfate side chains.

### The Transgenic Mouse as a Model System to Study Gene Function and Regulation

Two important aspects of cancer: the ability of malignant cells to escape destruction by chemotherapy, and the role of oncogenes and growth factors in the development of cancer are being investigated. Transgenic mice which contain a human cDNA (MDR1) encoding a multidrug transporter (P-glycoprotein) capable of conferring multidrug resistance in cell culture. Expression of the human MDR1 gene was detected in the normally drug-sensitive bone marrow of several lines of transgenic mice. Since bone marrow suppression is a major drawback to the use of chemotherapy, these transgenic mice will be used as a model to determine if higher doses of drugs can cure previously unresponsive cancers. The epidermal growth factor (EGF) receptor is the cellular homolog of the avian erythroblastosis virus erbB oncogene product. To elucidate the role of the EGF receptor and its ligands in growth control and transformation, mice bearing either the human EGF receptor gene or the human TGF $\alpha$  gene were produced. In both cases the transgene was expressed in a wide variety of mouse tissues. Studies are underway to determine the effects of inappropriate expression on the developing mouse.

### Regulation of the gal Operon of Escherichia coli

Gal repressor protein negatively regulates the expression of the gal operon of E. coli. Repression requires binding of repressor to two spatially separated operator sites, OE and OI. Previously it was shown by Dr. Sankar Adhya and his colleagues that occupation of the two operators sites by repressor is not sufficient to achieve normal repression, and it was proposed that an interaction between two operator bound dimeric repressors, giving rise to a DNA loop, is essential for repression. Recent experimental results strongly support this model.

It was also discovered that in a cell devoid of Gal repressor, a derepressed gal operon can be further induced two- to three-fold by the addition of the inducer, D-fucose. In vivo operator titration assays suggest that the gal operon is subjected to a new negative control under conditions when the classical Gal repressor is removed from the cell.

## Genetic Regulatory Mechanisms in Escherichia coli and Its Bacteriophage

Cyclic AMP binds to its receptor protein CRP, and induces a conformational change (allosteric transition) in the protein. The cyclic AMP.CRP complex, but not free CRP, binds at or near many E. coli promoters in a sequence-specific way and either represses or activates transcription. Several classes of mutations in the crp gene were isolated and characterized which identify the amino acids participating in the cyclic AMP-induced allosteric shift in the protein. On the basis of these results and the three-dimensional structure of the protein, it is proposed that cyclic AMP brings about a hinge reorientation to release the DNA-binding  $\alpha$ -helices; alignment between two subunits; and an adjustment between the position of the two domains in each subunit. Mutants have been obtained that are in an allosterically frozen conformation that is uninducible by cAMP. These mutations impart a defect in DNA binding and/or in transcription activation, and these data suggest that a cAMP-induced allosteric change is necessary not only for DNA binding but also for CRP-RNA polymerase interaction. The CRP-RNA polymerase interaction when bound to DNA is also being studied. Results suggest that a specific DNA distance between the two bound proteins is required for productive interaction. It is likely that there is a complicated arrangement between the two sites, in terms of DNA sequence and distance.

## Bacterial Functions Involved in Cell Growth Control

The role that protein degradation plays in regulating cell growth control is being investigated by Dr. Susan Gottesman's laboratory through the study of the proteases which carry out ATP-dependent proteolysis in E. coli. The Lon protease has previously been shown to degrade RcsA, a positive regulator of capsule synthesis, and Sula, a cell division inhibitor. The analysis of capsule synthesis is being continued. RcsC, a membrane protein, probably functions to sense environmental signals and transmits this information to the positive regulatory protein RcsB. RcsA may act directly on the regulated genes, or indirectly by affecting RcsB-RcsC interactions. The Clp protease, a second major ATP-dependent protease, has an ATP-binding subunit (ClpA) which shares homology with a family of proteins in both eukaryotic and prokaryotic cells. The protease subunit of the Clp protease (ClpP) contains a serine active site, and has only limited activity in the absence of ClpA. Clp is induced anaerobically; one target of Clp degradation is excess ClpA subunit. A third energy-dependent protease, the Alp protease, is capable of substituting for Lon under some conditions.

## **LABORATORY OF BIOCHEMISTRY**

The research activities of the Laboratory of Biochemistry, directed by Dr. Claude Klee, are primarily focused in two areas: the control of gene expression (where the emphasis is on the isolation and characterization of trans-acting regulatory proteins), and the role of proteins in cellular regulation.

## The Control of Gene Expression

Dr. Carl Wu's group has continued to study sequence-specific DNA-binding proteins in Drosophila, focusing on those binding at the heat shock genes and

the segmentation gene fushi tarazu, ftz. A major achievement this year was cloning of heat shock gene transcriptional activator, or Heat Shock Factor, HSF. Another important finding was that HSF, which exists in an inactive conformation, can be activated in vitro by exposure to heat, low pH, or an antibody raised against the active form of HSF, suggesting that a conformational change may be responsible for heat activation. It is hoped that elucidation of the nature of this structural change will provide insight into the mechanism by which this key signal transducer senses environmental stress and transmits the signal to the transcriptional machinery. The heat shock gene will be used as a model to study the cooperative or competitive interactions between nucleosomes and transcription factors by in vitro reconstitution experiments. These studies may help to clarify the role of histone proteins in controlling gene activity in higher cells. The second project deals with the regulation of gene expression allowing temporal and spatial expression of the ftz gene during Drosophila development. A DNA-binding factor that interacts with the ftz gene, ftz1, has been isolated. The function of ftz1 has been studied in vivo in transgenic flies and shown to be involved as a positive transcription factor in specific segments of the early embryo.

Another aspect of regulation of gene expression during development has focused on regulation of genes associated with muscle development and on characterization of the genes involved in myogenic determination. Dr. Bruce Patterson and his colleagues previously identified cis-acting elements involved in the developmentally regulated expression of muscle specific genes. They have now shown that the regulated expression of  $\alpha$ -actin and some myosin light chain genes in muscle is the result of a complex interplay of negative and positive regulatory proteins binding at the same DNA regulatory regions. The suppression of the  $\beta$ -actin gene in differentiated muscle is controlled by a cis-acting element in the 3' noncoding region of the gene. This element imparts the  $\beta$ -actin regulatory pattern when inserted, in either orientation, within the transcriptional unit of any gene expressed in a muscle cell background. A related and exciting development is the isolation of the CMD1 gene which is involved in the determination of the myogenic phenotype. BUDR, a thymidine analog that inhibits myogenesis, also inhibits the expression of CMD1 and may explain how the drug blocks differentiation. Expression of CMD1 in BUDR-treated cells will activate a cotransfected muscle-specific promoter and, in some cases, the endogenous muscle genes. The isolation and mode of action of the CMD1 gene product has become a major concern for this group.

This year Dr. Dean Hamer and his colleagues have made an important breakthrough in their understanding of how copper regulates metallothionein gene transcription in yeast. They discovered that copper binds directly to a bifunctional transcription factor, ACE1, and alters the conformation of a "DNA-binding domain" such that it can bind to DNA. Once the factor has bound to the metallothionein gene control sequences, a separate "activation domain" interacts the general transcription factors and thereby accelerates the rate of transcription initiation. The copper and DNA binding domain of ACE1 represents a new type of DNA recognition motif which they call the "copper fist". In higher eukaryotes, good progress has been made on isolating a mouse metallothionein gene regulatory factor, and the gene for an unusual zinc finger protein, also involved in the regulation of expression of the metallothionein gene, has been cloned and characterized.



The role of DNA methylation in the modulation of gene expression has become the main focus of interest in Dr. Ed Kuff's laboratory. For many years his group has been studying the regulation of an envelope-deficient mouse retrovirus, the intracisternal A-particle (IAP). IAPs are encoded by members of a multicopy family of endogenous proviral elements. IAP elements are transposable in the mouse genome and are multigenic. They are expressed in mouse embryos, some normal adult tissues, and many transformed mouse cells and represent a good model system to study gene regulation. Expression requires demethylation of sequences within the IAP 5' LTR along with a constellation of trans-acting cellular factors. The genetic basis for selective demethylation of IAP provirus is being investigated in normal mouse lymphocytes and pancreatic b cells, while relevant transcription factors are being studied in nuclear extracts of various transformed rodent and primate cells.

### Proteins and Control of Cellular Processes

The mechanism of stimulus response coupling mediated by  $Ca^{2+}$  and calmodulin remains the focus of interest in Dr. Claude Klee's laboratory. In collaboration with Kathen Beckingham (Rice University) they have used calmodulin mutants deficient in  $Ca^{2+}$  binding at either the low or high affinity  $Ca^{2+}$ -binding sites to study the cooperative binding of  $Ca^{2+}$  to the two halves of calmodulin. The same mutants were used to demonstrate that  $Ca^{2+}$  binding to all four sites of calmodulin plays a role in the activation of calcineurin, a calmodulin-stimulated protein phosphatase. A major achievement during the last year was the isolation and sequencing of two different cDNA clones of the catalytic subunit of calcineurin. The deduced amino acid sequences of the two proteins have allowed the characterization of the calmodulin-binding domain, the identification of a novel structural domain needed to maintain enzyme activity and revealed the extensive similarity of its catalytic domain with the catalytic subunits of protein phosphatases 1 and 2a, thereby defining a family of related protein phosphatases.

Dr. Shelby Berger and her colleagues are studying the role of prothymosin  $\alpha$  in cell division. The human prothymosin  $\alpha$  gene family has been cloned, and sequenced. It consists of one functional gene, and five processed pseudogenes. The gene gives rise to two mRNAs by a novel form of alternate splicing. Functional studies on prothymosin  $\alpha$  mRNAs, synthetic and natural proteins, and transfected cDNA and genes are not consistent with the extracellular role for thymosin  $\alpha$  proposed by others. Preliminary evidence suggests that prothymosin  $\alpha$  is required intracellularly for cell division. This group is also investigating the mechanism of action of the ribonuclease H of two viral reverse transcriptases. Contrary to previously held views these ribonucleases are endonucleases. They have identified a specific inhibitor of the viral enzyme.

Dr. Beverly Peterkofsky has continued to make steady progress in her study of the regulation of the synthesis of the extracellular matrix components, collagen and proteoglycan. Her previous proposal that the decreased synthesis of these matrix components in ascorbate-deficient guinea pigs is not related to the role of ascorbate in the hydroxylation of proline in collagen but to changes in circulating factors induced by fasting is supported by the demonstration that sera from scorbutic and fasting animals (ascorbate supplemented) have reduced levels of insulin-like growth factor (IGF-I) and also contain an

inhibitor of IGF-I. This inhibitor has now been shown to be a low molecular weight IGF-I-binding protein. As part of their study of the alteration of collagen synthesis in transformed cells Dr. Peterkofsky and her colleagues have shown that the defect in normal collagen synthesis in transformed cells is due to the loss of expression of the gene for the precursor of one of the two subunits of the type I collagen, accompanied by post-translational modification of the precursor of the other subunit.

### DNA Replication

Several lines of inquiry have been pursued in studies of P1 plasmid maintenance by Drs. Michael Yarmolinsky and Dhruva Chatteraj. Biochemical studies have shown the importance of steric factors in (1) the control of plasmid replication by DNA sites that bind the plasmid initiator protein and (2) the efficient functioning of the P1 centromere. Genetics studies have revealed the involvement of several heat shock proteins in plasmid replication. Physiological studies of the effects of interference with plasmid replication functions have led to a detailed analysis of the P1 lytic replicon and of P1 genes involved in bacterial addiction to the presence of the plasmid.

The earlier success in cloning a cDNA of human  $\beta$ -DNA polymerase by Samuel Wilson and his colleagues has been followed by the characterization of the core promoter for the human gene and the identification of a palindromic sequence GTGACGTCAC as a critical promoter element. A transcription factor that binds specifically to the palindromic sequence was purified and characterized. The preferential expression of this enzyme in testis and its induction by treatment with mutagens may shed additional light on its physiological role. The rat DNA polymerase  $\beta$  and the A1 hnRNP single strand-binding protein were over expressed in E.coli and their domain structure studied by limited proteolysis. The nucleic acid binding properties of the individual domains are under study. The determination of the kinetic parameters of the polymerization reaction catalyzed by HIV reverse transcriptase is a first step in an attempt to identify specific inhibitors of the enzyme.

### The Organization of the Human Genome

The panel of human-rodent somatic cell hybrids previously isolated and characterized by Dr. Wesley McBride continues to be an asset for many investigators at NIH and elsewhere wishing to determine the chromosomal localization of human genes. About 20-25 genes have been mapped during the past year, including 20 genes on 7 different human chromosomes. The role of several of these candidate genes in specific hereditary diseases will be evaluated.

Observations made this year in Dr. Maxine Singer's group extend earlier work on the expression of LINE-1 moveable elements in the human genome. They demonstrated that DNA sequences essential to the promoter activity of the 900 bp long 5'-leader segment of human LINE-1 elements lie within the first 100 bp at the 5' end. Moreover, LINE-1 promoter activity is about 100-fold higher in human teratocarcinoma cells than in HeLa cells. This finding is consistent with the earlier observation by this group that full length cytoplasmic polyA<sup>+</sup> LINE-1 RNA was detectable in teratocarcinoma cells but not in HeLa or other human cell types. The teratocarcinoma cell RNA was shown to be a mixture of transcripts from a large number of human LINE-1 elements. In vitro experiments

have shown that at least several of these RNAs can be messenger RNAs, yielding relatively large amounts of the product of the first open reading frame. Preliminary data suggest that the relatively low level of translation of the second open reading frame depends on reinitiation of translation rather than suppression of the stop codons that separate the two frames.

## **LABORATORY OF CELLULAR ONCOLOGY**

The Laboratory of Cellular Oncology, directed by Dr. Douglas Lowy, plans and conducts fundamental research on the cellular and molecular basis of neoplasia. Tissue culture cell systems and animal models are employed to study the induction and maintenance of benign and malignant neoplasia and reversal of the neoplastic state. Structure-function correlations are determined through detailed examination of individual genes which have been implicated in cancer.

### Tumor Gene Expression in vitro and in vivo

Oncogene studies have involved ras encoded proteins and the EGF receptor. A major problem of ras biology is to determine how the protein is stimulated to become biologically active and to identify the target(s) with which ras proteins interact. In a series of collaborative studies, Dr. Lowy's group has identified an effector region that apparently interacts with the putative ras target, they have found that a cytoplasmic protein called GAP (for GTPase activating protein) interacts with the ras effector domain. This result raises the possibility that GAP may be a ras target. The ability of GAP to inactivate normal ras encoded protein, and the result of additional studies now suggest that GAP may be only a negative regulator of ras.

### Analysis of Papillomaviruses

The strong association between certain papillomavirus (PV) types and specific human cancers, in particular cervical cancer, led to investigations of the biological activities of papillomavirus genes and the biochemical activities of their encoded proteins. Bovine papillomavirus (BPV) has served as a model for studying papillomaviruses because the viral genome readily transforms and replicates autonomously in rodent cell lines. E5 is one of the two major transforming genes of BPV. E5 encodes a 44 amino acid protein that is associated with the external surface of cytoplasmic membranes. Given its small size and location, it seemed unlikely that E5 would have intrinsic enzymatic activity, and investigations have been focused on the possibility that it induces transformation by activating other proteins involved in cell proliferation and transformation. It has been determined that E5 can potentiate the transforming activity of two growth factor receptors, the c-erbB and c-fms proto-oncogenes but not two other tyrosine kinases, c-src or c-fes. Potentiation can occur in either the presence or absence of exogenous ligand. In E5 containing cells, the EGF receptor is partially activated in the absence of exogenous ligand, and the half life of both ligand stimulated and unstimulated receptor is increased. From these results, it was hypothesized that E5 induces transformation by inhibiting the down regulation of activated growth factor receptors.

Since the HPV16 E6 and E7 genes are selectively retained and expressed in HPV16 and HPV18 associated cervical carcinomas, Dr. Lowy's group has undertaken an



in vitro analysis of their function and products. Biochemically, they found both E6 and E7 of HPV18 and of BPV can bind zinc, presumably through conserved cys-x-x-cys motifs. In BPVE6, they found that these motifs are essential for biological activity and for normal sub-cellular localization of the protein to the nuclear matrix. For HPV16, E7, but not E6, was found to transform NIH3T3 cells. E7 could also extend the lifespan of normal human foreskin keratinocytes, but E6 was required in addition for immortalization of the cultured keratinocytes. The results with human keratinocytes provide strong support for the hypothesis that the preferential maintenance and expression of E6 and E7 in human cervical carcinomas has pathogenic significance.

## LABORATORY OF PATHOLOGY

The Laboratory of Pathology, directed by Dr. Lance Liotta, is responsible for all the diagnostic services in anatomic pathology for the Clinical Center of the NIH and has a fully accredited 4-year residency program in anatomic pathology. A major focus of experimental research within the laboratory is a study of the molecular genetics of tumor cell invasion. Knowledge gained from these studies has led to the development of new therapeutic approaches designed to inhibit the development of cancer metastasis.

### Tumor Invasion and Metastases

A set of gene products has been identified in Dr. Liotta's laboratory which may regulate tumor invasion and metastasis: a) laminin receptor; b) autocrine factor; c) NM23 metastasis suppressor protein, d) type IV collagenase, and e) CSC-21 metalloproteinase inhibitor. These metastasis-associated gene products may be different from those which regulate tumor growth, and this is in keeping with the concept that benign tumors may grow in an unrestrained fashion, but fail to invade.

The laminin receptor is a cell surface protein which binds laminin, and is augmented in certain aggressive human cancers compared to non-malignant counterpart cells. Dr. Mark Sobel has cloned and sequenced the full-length cDNA for the laminin receptor. A recent discovery is that estrogen and progesterone regulate expression of the laminin receptor gene in breast cancer cell lines. These observations of augmented levels of cell surface laminin receptors in human tumors led to the development of a new therapeutic strategy; using anti-laminin receptor antibodies to target adriamycin encapsulated liposomes to tumor cells. In vitro studies using this approach demonstrated more than a ten-fold higher rate of killing of human breast carcinoma cells compared to breast epithelial cells. Clinical trials using this approach are in the preclinical toxicology phase.

The autocrine motility factor (AMF), a new cytokine secreted by tumor cells, binds with high affinity to a cell surface receptor and profoundly stimulates locomotion. Dr. Elliott Shiffmon's group found that a pertussis toxin sensitive G protein is required for the transduction of the AMF signal through the phosphoinositol pathway. Screening of inhibitors blocking this pathway has led to the identification of a Merck compound which may be of therapeutic value in cancer treatment. Preclinical toxicology studies have been completed and clinical trials are planned.

A recently published clinical study describes the measurement of AMF in the urine of patients with transitional cell carcinoma of the bladder. A very strong correlation was found between the AMF levels and tumor grade, stage of invasion, and recurrence. Wider clinical testing is underway to determine the efficacy of this test in the early detection of bladder cancer recurrence.

NM23 is a gene expressed to a higher level in low metastatic tumor cells compared to high metastatic cells. It encodes a putative metastases suppressor protein. A full-length NM23 cDNA clone has been sequenced. The encoded protein sequence has properties suggesting a leucine-zipper DNA binding protein. The NM23 protein has a size of 17 kDa. The NM23 protein is virtually identical to a *Drosophila* gene (*awd*) product which plays a critical role in morphogenesis and tissue pattern formation. The discovery of NM23 therefore provides a link between development and metastasis. The negative correlation of NM23 expression with metastases was confirmed in four different animal model systems using multiple clones. A strong negative correlation was found between human breast carcinoma aggressiveness, in terms of lymph node metastases, and NM23 mRNA expression. Preliminary data suggests that the NM23 gene location may be on chromosome 11, possibly on the short arm region shown by Callahan to be deleted in more aggressive human breast cancer cases. Additional preliminary data has demonstrated a strong suppression of experimental metastases in a series of tumor cell clones transfected with NM23 compared to a series of control transfections. NM23 may provide a number of significant prognostic and therapeutic strategies.

The structural backbone of the basement membrane is type IV collagen, a collagen genetically distinct from the other types of collagen. It was originally determined that type IV collagen was resistant to known mammalian collagenases. Dr. Liotta's group discovered type IV collagenase, a metalloproteinase which cleaves type IV collagen in a pepsin resistant site located 1/4 of the distance from the amino terminus of the substrate. The enzyme is secreted in a latent 70 kDa form which fails to degrade type IV collagen. When the enzyme is activated, it becomes reduced to a molecular size of 62 kDa and can specifically cleave type IV collagen. Both latent and active forms of this enzyme are gelatinolytic. This enzyme is secreted in large amounts by tumor cells and its rate of elaboration is correlated with the metastatic phenotype in several systems.

Affinity purified antibodies made against synthetic peptides corresponding to the latent versus active enzyme were used to study the expression of the enzyme antigen during progression from human in situ to invasive breast carcinoma. The latent type IV collagenase was found associated to a low degree with benign duct myoepithelial cells. In contrast, the active enzyme form was not present in normal ducts or benign ductal proliferation, was present variably in comedo carcinoma in situ, and was markedly augmented in primary invasive carcinoma and metastases. This indicates that enzyme activation may be an important correlate of tumor progression. Type IV collagenase production also shows a strong correlation with invasive grade in human colorectal cancer, and is markedly augmented following ras oncogene transfection in a variety of murine and human cell types.

Analysis of the full-length cDNA clone encoding the human latent type IV collagenase has revealed three separate functional domains, which suggests

three new strategies for inhibition of the enzyme: a) blocking activation of the latent metalloproteinase, b) blocking binding of the latent or active metalloproteinase to the substrate, and c) inhibition of the active (cleavage-producing) site of the enzyme. Specific synthetic peptides have been developed which accomplish all three strategies.

The first clinical application of this information is in the diagnosis and prognosis of cancer. Immunohistologic staining using antibodies to various domains on type IV collagenase have proven to be a useful means to judge the invasive potential of a tumor and to identify micrometastases in lymph nodes or other tissues distant from the primary tumor site. Type IV collagenase may be a particularly good marker in urine for bladder cancer, and may also prove useful as a serum marker for systemic cancers. Preliminary data indicate that levels of type IV procollagenase mRNA and type IV procollagenase gene rearrangement and amplification may be of prognostic value in human carcinomas.

The second clinical application is therapy. Synthetic, natural, or recombinant inhibitors of type IV collagenases may have significant therapeutic potential as agents which block angiogenesis and tumor invasion. They may have low toxicity since basement membrane turnover is rare in normal tissues. There also may be therapeutic utility for the recombinant enzyme itself or for enzyme fragments.

During the past year, a novel proteinase inhibitor was discovered which binds with a 1:1 stoichiometry to type IV collagenase. The inhibitor has been purified to homogeneity and the complete primary structure has been determined. The novel inhibitor, termed CSC-21, is the first new member of the TIMP (tissue inhibitor metalloproteinases) family with conservation of all 12 cysteine positions. This new inhibitor can be considered in the class of tumor suppressor gene products.

### Hematopathology

An active research program is carried out on the immunological characterization of malignant lymphomas. This information is utilized to study the relationship of malignant lymphomas to the normal immune system, to develop improved classifications of disease, to distinguish new clinicopathologic entities, and as a basis for immunotherapy.

Dr. Jeffrey Cossman has been studying the molecular basis of the diagnosis of human lymphomaproliferative disease. This work has already produced a number of major findings. The T- and B-cell lineage of diffuse, aggressive lymphomas as determined by phenotypic studies can be confirmed by detection of appropriately rearranged T-cell receptor or immunoglobulin genes. Moreover, in some patients' tissues which lack evidence of malignant lymphoma by conventional histology or immunologic studies, malignant lymphoma can be identified by these techniques.

Dr. Mary Alice Stetler-Stevenson has demonstrated that frequent relapse of follicular lymphoma, the major obstacle to cure, is a consequence of clonal expansion of daughter cells derived from a common stem cell. Thus, despite the "clinical" remission achieved by therapy in most patients, residual lymphoma cells must persist. To detect occult lymphoma, investigators in this



laboratory have specifically amplified the joined bcl-2/JH DNA sequences created by the t(14;18) translocation seen in nearly all follicular lymphomas. Using multiple rounds of primer-directed DNA polymerization (polymerase chain reaction, PCR), one can detect a single copy of bcl-2/JH, which is four orders of magnitude more sensitive than flow cytometry or Southern blot restriction analysis. Genomic DNA sequence analysis of four lymphomas confirmed that the size of the amplified fragment serves as a unique tumor marker. Direct application to clinical samples has demonstrated lymphoma cells which were otherwise undetectable.

### Gene Regulation

Dr. David Levens has developed a novel method for identifying sequence-specific DNA binding proteins. The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. These same proteins also bind to a negative cis-element approximately 330 bp upstream of c-myc promoter P1. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not in itself possess full specificity. The second component does not in itself bind DNA, but upon forming a complex with the first component confers greatly enhanced power to discriminate between different sequences.

Application of an exonuclease assay to the c-myc has revealed multiple cis- and trans-elements both upstream and downstream of the c-myc promoter P1. Current studies have focused on 3 regions, each of which interacts with one or more sequence-specific binding proteins. A negative cis-element present in intron one of the c-myc gene is frequently mutated in Burkitt's lymphoma.

### **DERMATOLOGY BRANCH**

The Dermatology Branch, directed by Dr. Stephen Katz, conducts both clinical and laboratory research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin, and the host's response to these diseases. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center.

### Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases

Dr. Katz's laboratory has continued to study the immunological functions of cells of the epidermis. When murine 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. Utilizing these cells in primary immune responses in vitro, they found that the T cells responded preferentially to the hapten to which they were "primed." T cell lines have been produced from these in vitro sensitized cells. During the first few stimulations the primed T cells produce mainly IL-2, however after multiple stimulations they produce mainly IL-4 and not IL-2. Utilizing this in vitro

sensitization system, they have attempted to generate primary in vitro responses to tumor-associated antigens such as the Friend Leukemia Virus associated gp-70 protein, but have been unable to detect any antigen processing or presentation by cultured or freshly prepared epidermal Langerhans cells. Preliminary studies demonstrate that cultured human Langerhans cells exhibit increased class II MHC molecules and are potent alloantigen presenting cells. Dr. Katz's group plans to assess the ability of these cells to present protein antigens and then will attempt to generate primary in vitro responses to viral- or tumor-associated antigens.

Studies assessing the effects of cyclosporine A (CSA) on antigen presenting and accessory cell functions of Langerhans cells have demonstrated that offers a 2 hour incubation with CSA, Langerhans cells become relatively unable to perform many of the T cell stimulatory functions. In addition, CSA has a profound effect on the proliferation of epidermal cells. Dr. Katz and his colleagues have also studied the effects of dexamethasone on epidermal Langerhans cells. From thier in vitro studies, it appears that dexamethasone is cytolytic to a subpopulation of Langerhans cells. In studies using various activation signals, it is clear that phorbol and ionomycin activation of T cells cannot be inhibited by dexamethasone, whereas it is completely blocked by cyclosporine.

#### Molecular Basis of Autoimmune Skin Diseases

Dr. John Stanley's group is studying autoantibody-mediated skin diseases in order to learn more about the pathophysiology of these diseases, and also the structure and function of normal epidermis and epidermal basement membrane zone. Specifically, antibodies in these diseases define molecules in the normal epidermis. Dr. Stanley has characterized the antigens defined by antibodies in the sera of patients with three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV) and pemphigus foliaceus (PF). He has also determined, with immunochemical methods, that the BP antigen is a 230 kD protein with a pI of 8. The sera of almost all patients with BP bind to this polypeptide, and, therefore, immunoprecipitation can be used as a diagnostic technique. A cDNA clone encoding the C-terminal end of this antigen has been isolated which is being used as a probe to find the full length cDNA for BP antigen. The encoded peptide is not homologous to any known protein.

The PV and PF antigen complexes extracted from normal human epidermis have also been characterized, and shown to contain the adhering junction molecules desmoglein (PF antigen) and plakoglobin (both antigens). Thus, in diseases of defective cell adhesion, autoantibodies are directed against adhering junction components.

#### Molecular and Cell Biology of Immunoreactive Molecules in the Skin

A new research program has been initiated with a primary goal of elucidating the molecular mechanisms regulating the expression of immunoreactive molecules in the skin. ICAM-1, or intercellular adhesion molecule-1, is a cell surface protein that is the major ligand for LFA-1, or lymphocyte function associated antigen-1, an anchoring receptor on all T cells whose interaction with ligand is critical for proper T-cell function. Cutaneous T-cell inflammatory and neoplastic diseases may depend upon the regulation of ICAM-1 expression by

various cells in the skin. Dr. Wright Caughman has shown that human keratinocytes (HK) in culture do not normally express ICAM-1, and that exposure of HK to human interferon-gamma (IFN) or tumor necrosis factor-alpha (TNF), two biologic response modifiers (BRM), results in the rapid induction of ICAM-1 mRNA expression, protein biosynthesis, and surface expression. Human dermal microvascular endothelial cells (HDMEC) constitutively express ICAM-1, and that this expression can be rapidly upregulated both at the level of mRNA and in surface expression by exposure to human interleukin-1 (IL-1). Recent studies showed specific T-cell binding to HDMEC in vitro is dependent upon ICAM-1 expression by HDMEC and can be upregulated as well by factors which increase ICAM-1 expression.

ICAM-1 expression in HK and HDMEC is regulated at the level of gene transcription. A 19 kb genomic clone has been isolated that contains portions, if not all, of the ICAM-1 gene. Mapping and sequencing of the 5' flanking regulatory region of this genomic clone of ICAM-1 has been partially completed.

#### Therapy of Skin Cancer and Disorders of Keratinization

Dr. Gary Peck's laboratory is 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 as well as in the prevention of cancer.

#### **METABOLISM BRANCH**

The Metabolism Branch, directed by Dr. Thomas Waldmann, conducts laboratory and clinical research with the ultimate goal of defining host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations are carried out in patients with immunodeficiency diseases that are associated with a high incidence of neoplasia, as well as in patients with malignancy, especially T- and B-cell leukemias.

#### Molecular Analysis of Translocations of Transforming Genes to the Locus of T-cell Antigen Receptor Genes

Previously, analyses of immunoglobulin and T-cell receptor gene rearrangements were used to define the lineage (T or B cell) of cells lacking conventional markers, to establish whether abnormal lymphocytic populations are polyclonal, oligoclonal or monoclonal, and to broaden the scientific basis for the diagnosis and monitoring of the therapy of lymphoid neoplasia. More recently, Dr. Waldmann has exploited illegitimate chromosomal translocations in T cell leukemia involving the T cell receptor genes to clone elements involved in the regulation of normal and malignant cell proliferation. A putative transforming gene has been identified (SCL-1) that is expressed in hematopoietic tissues. The gene was identified through the molecular cloning of a translocation between chromosomes 1 and 14;t(1:14)(p33;q11) that occurred in a human stem cell leukemia that was able to differentiate into myeloid and lymphoid lineages. As a consequence of the translocation, a novel fusion transcript was generated between the sequences on chromosome 1 and chromosome 14. The gene of chromosome 1 manifested a DNA binding motif shared with the *Drosophila* daughterless gene and the *myc* and MyoD proteins. This gene is expressed in fetal liver and normal bone marrow and may be important for hematopoietic stem cell development and oncogenesis.



## Molecular Analysis of Trans-Acting Factors That Mediate Lymphoid Specific Gene Transcription

As an approach to understanding development in the B lymphocyte lineage, Dr. Louis Staudt has characterized trans-acting factors that mediate lymphoid-specific gene transcription. The lymphoid-specific transcription of immunoglobulin (Ig) genes has been chosen as a model. The promoters of all Ig variable genes and the Ig heavy chain enhancer contain a critical octamer DNA motif, ATTTGCAT. This motif, together with a TATA motif, is necessary and sufficient for lymphoid-specific promoter activity. Nuclear extracts of lymphoid and non-lymphoid cell lines have been assayed for proteins which specifically bound to the octamer motif, and two major species were detected: a ubiquitous octamer binding protein (Oct-1) and a lymphoid-specific octamer binding protein (Oct-2). Oct-2 functioned as a transcription factor in vitro using immunoglobulin promoter templates. Dr. Staudt cloned the human Oct-2 structural gene by screening a phage  $\lambda$ gt11 expression library with a radio-labeled DNA probe containing the octamer motif. The cloned gene encodes an octamer-specific DNA binding protein which is itself lymphoid-restricted in expression.

By sequencing the Oct-2 gene Dr. Staudt identified a 60 amino acid domain that is homologous to the "homeo box" domain of proteins which are important for cell type determination and development in lower eukaryotes. This domain of the Oct-2 protein was shown to be required for DNA binding by site directed mutagenesis. This represents the first demonstration that a mammalian gene containing a homeo box domain is a sequence-specific DNA binding protein. The presence of the homeo box in the Oct-2 gene lends support to the notion that this gene is important for lymphoid development. Full length Oct-2 cDNA clones revealed additional regions of homology with other transcription factors. One highly conserved 75 amino acid domain, the "POU" box, is shared by a subset of homeo box proteins that includes a pituitary-specific human transcription factor, Pit-1. In addition, Oct-2 has a "leucine zipper" domain which it shares with the nuclear oncogenes *myc*, *fos* and *jun* and with other transcription factors. The leucine zipper may mediate the interaction of Oct-2 with other regulatory proteins. Alternative splicing of the Oct-2 gene generates a variety of distinct Oct-2 proteins, one of which lacks the leucine zipper and thus might be functionally unique.

Recent evidence indicates that the octamer motif may control the expression of other lymphoid-specific genes besides Ig, notably the Bcl-2 proto-oncogene involved in the pathogenesis of human follicular lymphomas. Using the cloned Oct-2 gene, it should be possible to determine the range of genes which are expressed exclusively in lymphocytes under the transcriptional control of the octamer motif. Recent evidence suggests that oct-2 may be involved in the expression of T cell activation genes.

## Somatic Gene Therapy for Human Genetic Disease

Dr. Michael Blaese and his collaborators, Steven A. Rosenberg and W. French Anderson, have now performed the first authorized clinical experiment in which a foreign gene has been introduced into humans. A recombinant murine retroviral vector containing the bacterial gene encoding Neomycin resistance (*neoR*) was used to label tumor infiltrating lymphocytes (TIL) used in the treatment of patients with malignant melanoma. The next phase of these studies will involve

the insertion of genes encoding various cytokines into TIL in an attempt to improve their tumoricidal activity for a variety of tumors. Dr. Blaese prepared vector constructs with the alpha and gamma interferon genes as well as genes for tumor necrosis factor (TNF) and IL-1 $\beta$ . These vector preparations are currently being characterized for their ability to produce these cytokines in both lymphoid and non-lymphoid cells. Major studies of the biological effects of cells constitutively secreting these molecules are also underway as are studies of the potential utility of using lymphocytes as cellular vehicles for gene therapy of non-malignant diseases such as adenosine deaminase (ADA) deficiency. T lymphocytes from SCID patients have been grown to high numbers using anti-CD3 and rIL-2 stimulation and the human ADA gene has been successfully introduced into these cells. A clinical protocol for the treatment of ADA(-)SCID with gene-treated cultured T cells is now being prepared for submission to the Recombinant DNA Advisory Committee.

Dr. Blaese has also continued his work with the compound Succinylacetone (SA) which appears to have potent immunosuppressive properties. SA greatly prolongs skin allograft survival in rats and permits indefinite survival of totally mismatched cardiac allografts in rats.

#### The Genetic Control of the Immune Response: Application to the Design of Synthetic Vaccines

Dr. Jay Berzofsky has been studying the mechanisms by which T cells recognize antigens on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, and the application of these principles to the design of synthetic vaccines for AIDS and malaria. Earlier studies in myoglobin demonstrated that different T cells can see the same determinant in different positions or conformations in the same MHC molecule. In addition, Dr. Berzofsky showed that peptides that can fold as amphipathic alpha helices are statistically more frequent among immunodominant T-cell determinants than in protein sequences in general.

Dr. Berzofsky applied these results to the design of synthetic vaccines for malaria and AIDS. In the malaria study, two immunodominant sites for human and mouse helper T cells were found. T cells from some humans from a malaria endemic-area of Africa could distinguish the variants of these sites from different isolates of P. falciparum, however, evidence was obtained for crossactivity among the variants of both sites, suggesting that it should be possible to develop a vaccine that may be useful for many strains of P. falciparum. In the case of AIDS, 6 multideterminant regions of the envelope protein have been identified that contain overlapping helper T-cell antigenic determinants seen by mice of different MHC types. Each region can be seen by most MHC types allowing circumvention of MHC restriction problems. Four of the peptides were studied in HIV-infected humans. About 80% of humans early in HIV infection lose the ability to respond in a T-cell proliferative response to soluble protein antigens, but about 50% retain a T-cell response to the same antigens as measured by IL-2 production. This finding may be of prognostic significance. Each peptide elicited in vitro T-cell responses in cells from 50-67% of unrelated HLA-diverse donors who retained the ability to respond to control antigens, and 85-90% of such donors responded to at least one of the 4 peptides, so many peptides may not be required for an MHC-diverse population. In addition, one of these peptides served as a very potent helper determinant when attached to a neutralizing antibody epitope to elicit neutralizing

antibodies. Because the CTL determinant previously identified in the envelope protein is highly variable among HIV isolates, Dr. Berzofsky examined the effect of this variability on T-cell recognition and found that the same site is a CTL determinant on several isolates of HIV. He has been able to identify residues that contribute to binding to class I MHC molecules, and one residue that seems critical for recognition by the T cell receptor. Thus, HIV can mutate residues that interact with MHC molecules. The identification of these helper and CTL determinants, approaches to circumvent MHC restriction, and the understanding of virus variability, should prove useful in the design of vaccines.

#### The Multi-Chain IL-2 Receptor: Chemical Characterization, Function, and Use as a Target for Immunotherapy

Antigen-stimulated activation of resting T cells induces the synthesis of interleukin-2 as well as high affinity IL-2 receptors that are not expressed on resting T cells. Dr. Waldmann previously identified two peptides that bind IL-2, the 55 kD peptide reactive with the anti-Tac monoclonal, and a novel 70/75 kD (p75) peptide. He proposed a multichain model for the high affinity IL-2 receptor in which both the p55 Tac and the p75 IL-2 binding peptides are associated in a receptor complex. Recently additional p22, p35, p40, p95 and p180 peptides have been identified that may be associated with the 55 kD Tac peptide in the multichain receptor.

IL-2 receptor represents an extraordinarily versatile therapeutic target. T cells in patients with certain lymphoid malignancies and select autoimmune diseases, as well as individuals rejecting allografts express the IL-2 receptors identified by the anti-Tac monoclonal antibody, whereas normal resting cells and their precursors do not. The unmodified anti-Tac monoclonal antibody has been utilized to block binding of IL-2 to its growth factor receptor, and has led to remissions lasting 1, 5 and over 8 months in the treatment of patients with adult T cell leukemia. This therapy has not had toxic complications and has also been used effectively in the prevention of early graft rejection in patients receiving donor renal allografts.

In order to improve the effectiveness of IL-2 receptor directed therapy, different approaches have been used to modify the antibody to augment its capacity as a cytotoxic agent. For example, in collaboration with Dr. Ira Pastan a genetically modified form of Pseudomonas exotoxin (PE40) has been developed from which the first domain (which is responsible for ubiquitous cell binding) was deleted. This truncated toxin was conjugated to anti-Tac or IL-2 in order to provide a method of delivering the toxin directly to the surface of IL-2 receptor expressing cells. Anti-Tac (Fv) sequences encompassing the variable regions of the anti-Tac light- and heavy-chains were genetically coupled to the truncated Pseudomonas exotoxin sequence to generate a single co-linear protein with the specificity of the antibody. In addition, alternative cytotoxic agents were developed by coupling radionuclides to anti-Tac. For example,  $^{212}\text{Bi}$ , an alpha-emitting or  $^{90}\text{Y}$  a beta-emitting radionuclide conjugated to anti-Tac by use of bifunctional chelates were effective and specific agents for the elimination of Tac-expressing cells and prolonging survival of cardiac allografts in animals. The use of mouse anti-Tac is hindered by the host immune response to the murine antibody and by the lack of effector cytotoxic capacity of the monoclonal. A "humanized" anti-Tac antibody has been constructed by placing the complementary determining regions (CDRs) of



the murine anti-Tac antibody into the human variable region framework and  $\alpha_1$  constant regions. In addition, a molecular model of the murine anti-Tac was used to identify the amino acids which although outside of the CDRs had a high probability of interacting with either the CDRs or the antigen surface. Such residues could have an effect on antigen binding and thus were retained in the constructed antibody.

Over the past year, hyperchimeric "humanized" anti-Tac antibodies have been generated in which the molecule is entirely human IgG1 kappa except for the small hypervariable segments of the complementarily determining regions retained from the mouse antibody. It is hoped that these "humanized" antibodies will be minimally immunogenic. Furthermore, they add the new functional activity of antibody dependent cellular cytotoxicity that was absent in the parent mouse anti-Tac while maintaining the important functions of high affinity binding and T-cell receptor blockade. Clinical trials will be initiated to define the efficacy of each of these agents in the treatment of patients.

#### **IMMUNOLOGY BRANCH**

The Immunology Branch, directed by Dr. David Sachs, carries out laboratory investigations in basic immunology with particular emphasis on studies of the major histocompatibility complex (MHC) and its role in transplantation in animal models and in man.

#### Development of Models for the Induction of Specific Tolerance to MHC Products

The induction of intentional mixed chimerism across MHC barriers has been investigated as a means of producing specific transplant tolerance. The use of mixtures of T-cell depleted syngeneic plus allogeneic bone marrow has been found to produce permanent specific tolerance with full immunocompetence. Reconstitution of irradiated animals with a mixture containing T cell depleted syngeneic marrow plus non-T-cell depleted allogeneic marrow has been found to produce complete allogeneic chimerism while avoiding graft-vs-host disease (GVHD). This model may be useful in the treatment of leukemia, since it does not appear to eliminate the graft-vs-leukemia (GVL) effect. In order to decrease the toxicity of ablative radiation regimens, monoclonal anti-T-cell subset antibodies have been substituted for part of the irradiation. Treatment with such antibodies can deplete mature T-cells effectively from all peripheral lymphoid tissues, but not from the thymus. This selective thymic irradiation has produced long-term mixed chimerism and specific tolerance across an MHC barrier with a non-lethal preparative regimen. This regimen has now been successfully applied to rat-mouse xenogeneic chimeras as well.

#### Studies of Bone Marrow and Organ Transplantation in Miniature Swine

Without exogenous immunosuppression, miniature swine matched for class II and transplanted across a class I plus minor antigen difference frequently develop specific systemic tolerance. The mechanism of this tolerance has been investigated in vitro and has been found to involve a specific depletion of class I reactive helper T-cell populations.

Because the response to ablative regimens in miniature swine is more similar to that of man, establishment of a bone marrow transplant regimen in this model

has been carried out as a pre-clinical step. Ablative radiation regimens have been established similar to those used for HLA identical sibling transplants in man, and have been found satisfactory to permit MHC matched bone marrow allografts. A new regimen with fractionated irradiation has been found necessary to permit successful transplantation of MHC mismatched bone marrow. This regimen is not sufficient to permit acceptance of T-cell depleted bone marrow grafts. Non-T-cell depleted bone marrow grafts across full MHC barriers were found to produce aggressive lethal GVHD. Addition of T-cell depleted autologous bone marrow to the non-T-cell depleted allogeneic inoculum has been attempted and, in some cases, has permitted engraftment while avoiding lethal GVHD. These data may have potential applicability to clinical transplantation.

The use of bone marrow transplants to induce tolerance to organ allografts in this model have been demonstrated. Kidney grafts matched for MHC with the bone marrow donor enjoy the same long-term survival as do matched kidney allografts

#### **EXPERIMENTAL IMMUNOLOGY BRANCH**

The Experimental Immunology Branch, directed by Dr. Alfred Singer, carries out laboratory investigations in basic immunobiology with particular emphasis on lymphocyte differentiation and regulation; T cell biology of the immune responses; transplantation biology; structure, regulation and function of genes involved in the immune response; and tumor immunology. A flow cytometry laboratory is maintained that supports over 80 individual research projects during the past year for investigators in the EIB and elsewhere at the NIH.

#### Lymphocyte Differentiation and Regulation

Dr. Alfred Singer's laboratory has examined the role of CD4 and CD8 accessory molecules in T cell differentiation and function. These studies have revealed that protein kinase C (PKC) activation results in the rapid internalization from the cell surface of T-cell receptors (TcR) and CD4, but does not result in the rapid internalization of CD8, suggesting that the CD4 and CD8 differ in their biochemical responsiveness as well as their ligand specificities for class II and class I MHC respectively. Studies with anti-CD4 monoclonal antibody (MAb) modulation of cell surface CD4 have revealed that anti-CD4 induced internalization of CD4 induces co-internalization of TcR both in vitro and in vivo. Interestingly, the co-internalization of TcR with CD4 is dependent either on PKC or a related intra-cellular mediator, as intra-cellular depletion of PKC disrupts the co-internalization of TcR induced by anti-CD4 cross-linking. During these studies, Dr. Singer found that antibody induced engagement of CD4 on immature CD4<sup>+</sup>8<sup>+</sup> (double positive) thymocytes caused the immature double positive thymocytes to markedly increase their expression of TcR by 3-5 fold, as opposed to the reduction in TcR expression that occurs upon antibody induced CD4 engagement on mature CD4<sup>+</sup>8<sup>-</sup> (single positive) thymocytes. Since increased TcR expression is a necessary step in the differentiation of immature double positive thymocytes into mature single positive thymocytes, these studies indicated a critical role in T cell differentiation for CD4 engagement on developing thymocytes. Dr. Singer's studies to elucidate the molecular basis for the increased expression of T cell receptor on immature thymocytes in response to CD4 engagement revealed that the great majority of receptor proteins are degraded within immature thymocytes, and that CD4 engagement results in a decreased rate of degradation. Thus, the increase in



TcR expression does not result from increased synthesis, but rather from decreased degradation.

The role of T cell receptor expression in thymocyte development has also been studied. In studies in mice which are genetically unable to express T cell receptor (SCID mice), it was observed that TcR<sup>-</sup> SCID thymocytes were arrested at the CD4<sup>-</sup>8<sup>-</sup> stage of differentiation. Interestingly, if TcR<sup>+</sup> cells were introduced into the SCID thymus, the SCID thymocytes would remain TcR<sup>-</sup> but would differentiate into CD4<sup>+</sup>8<sup>+</sup> cells. These studies indicated a role for functional TcR<sup>+</sup> cells in initiating the differentiation of TcR<sup>-</sup> CD4<sup>-</sup>8<sup>-</sup> thymocytes, a function that might be performed by TcR( $\alpha$ ) in normal T cell ontogeny.

If thymic selection occurs at the CD4<sup>+</sup>8<sup>+</sup> double positive stage of differentiation, immature double positive thymocytes must be responsive to membrane-initiated differentiation signals. Recent studies have revealed that double positive thymocytes will activate tyrosine kinases which tyrosine phosphorylate the zeta chain of the T cell receptor complex in response to cross-linking of cell surface CD3, CD4, CD8, and TcR( $\alpha$ ). Since most double positive thymocytes have their zeta chain phosphorylated *in vivo*, these results suggest that most double positive thymocytes have already received *in situ* membrane-initiated differentiation signals that have activated their intracellular tyrosine kinases.

Studies in the laboratory of Dr. Stephen Shaw have been directed at characterization of heterogeneity among subsets of human peripheral blood T cells and determining the functional capacities of the subsets defined. Their studies confirm and extend previous analysis of T cell subsets distinguished by different isoforms of CD45, which they have interpreted to be naive and memory T cells. Methodologic progress allows preparation of very pure subsets whose functional capacities have been studied. Naive cells have markedly reduced proliferative responses to various regimens of receptor-mediated activation; however, their capacity to respond is demonstrated by augmented response in the presence of co-stimuli such as that provided by IL1/IL6, CD28 or CD44. This suggests important regulatory mechanisms for co-stimuli *in vivo* in determining responsiveness of particular subsets. Systematic phenotypic analysis of CD4<sup>+</sup> cells with more than 200 monoclonal antibodies reveals unexpected heterogeneity which is being categorized by multicolor flow cytometry. Such integrated phenotypic and functional analysis is essential to adequately address the functional complexities in T cell responses.

The role of endogenous lymphokines in T cell activation has also been evaluated in Dr. Richard Hodes' laboratory. The proliferative responses of type 2 T helper clones are inhibited by antibody specific for the lymphokine interleukin 4 (IL4), which is produced by these clones. This demonstrates that IL4 acts as an autocrine growth factor for these T cells. In addition, mRNA levels corresponding to a number of activation genes are inhibited by the presence of anti-IL4 antibody during T cell activation. In contrast, the level of IL4 mRNA actually increases in the presence of this antibody, suggesting that endogenously produced IL4 may normally down regulate its own steady state mRNA levels.

Studies of T lymphocyte responses in HIV<sup>+</sup> asymptomatic individuals by Dr. Gene Shearer indicate that approximately 50% of these patients exhibit selective CD4<sup>+</sup> T helper cell functional defects without any AIDS symptoms and without

loss of CD4<sup>+</sup> cell numbers. A defect of antigen-presenting cell (APC) function was not observed in any such asymptomatic individuals. Many patients in various stages of disease exhibited a selective loss of CD4<sup>+</sup> T helper function but retained CD4<sup>-</sup> T helper function to HLA alloantigens. The results of this study: a) permit the detection of three stages of T helper dysfunction not previously defined by AIDS diagnostic tests; b) do not implicate an APC defect early in disease progression; and c) provide evidence for an alternate (CD4<sup>+</sup>) T helper pathway that may be utilized for developing immunotherapy strategies in HIV<sup>+</sup> patients.

### Cell Biology of Immune Responses

Dr. Stephen Shaw's laboratory has been identifying and characterizing the functions of cell surface molecules which facilitate T cell recognition ("accessory molecules"), particularly those which mediate adhesion. The functional role of ICAM-1, previously characterized as a cell surface ligand for LFA-1, has been studied not only in T cell adhesion but also as a costimulus for T cell activation. Its capacity to facilitate CD3-receptor mediated activation, together with other aspects of its regulation and function, suggest that it may be one of the most important "second signals" for T cell activation. Studies of other accessory molecules have identified CD44 as an additional member of the functionally important class of molecules involved both in T cell adhesion and activation. Studies of T cell/monocyte adhesion are characterizing the molecular basis of this immunologically important cell interaction.

Studies on the mechanism of lymphocyte-mediated cytotoxicity in Dr. Pierre Henkart's laboratory have shown that red blood cells are killed by both cloned cytotoxic T lymphocytes and helper T cells when linked to these cells by their T cell receptor. This cytotoxicity appears to operate by the same mechanism as the killing of more complex nucleated targets. Secretory granule proteases play an important role in mediating target cell DNA breakdown during killing by cytotoxic T lymphocytes (CTL). Recent studies showed that cytotoxicity mediated by purified granules from CTL or natural killer cells is accompanied by target DNA breakdown, and that this appears to require both cytolysin and another granule component. This component has been purified by assaying DNA release from detergent-isolated nuclei. In CTL granules this activity is identical to the previously described serine protease granzyme A. The mechanism by which this protease causes DNA breakdown is currently under investigation.

### Transplantation Biology

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL), may play a significant role in mediating allogeneic marrow graft rejection. Using a murine model system, it was found that: 1) a cloned CTL population was sufficient to reject an allogeneic marrow graft; and 2) the mechanism by which these marrow grafts were rejected was specific for MHC gene products expressed by the donor marrow and corresponded to the cytotoxic specificity of the clone. One approach to inhibit such graft rejection by CTL is by in vivo administration of monoclonal antibodies with specificity for CTL surface antigens. Administration of anti-CD3 to mice results in T cell activation within hours of administration and produces extramedullary hematopoiesis in the spleen. In considering the use of anti-CD3

results indicate that it may be beneficial to time the injection of allogeneic marrow so that it is present during the period of T cell activation induced by the antibody in order to provide the possible benefit of factors which stimulate hematopoiesis.

Studies in murine marrow chimeras have suggested that MHC mismatched marrow transplantation is associated with immunoincompetence of the T cell arm of the immune response. However, recent studies in a rhesus monkey model imply that the generation of T cells in marrow grafted primates may not be the same as in the mouse. Such differences would potentially have important implications for the transplantation of HLA-mismatched marrow in man.

In theory, in vivo allografts could either be destroyed by an antigen-non-specific lymphokine mechanism or by antigen-specific T-effector cells that assessed each individual cell in the graft for expression of alloantigens. To distinguish these mechanisms, Dr. Alfred Singer's laboratory constructed allophenic mice and assessed the antigen-specificity of the effector limb of the in vivo rejection response. These studies revealed that the effector mechanism of in vivo allograft rejection is highly antigen-specific, destroying cells expressing alloantigens but sparing cells within the graft that do not.

Somewhat surprisingly, Dr. Singer's laboratory found a similar degree of antigen-specificity for Ia-restricted CD4<sup>+</sup> T-effector cells, even though most of the epidermal cells within a skin allograft are MHC class II negative. It is likely from these findings that Ia<sup>-</sup> keratinocytes are induced to express Ia determinants during the in vivo rejection response, making them targets for Ia-restricted T-effector cells.

Graft-versus-host disease (GvHD) is dependent in its generation on the presence of immune competent T cells in the donor marrow with specificity for allogeneic antigens of the host. One approach to preventing GvHD is the removal of these T cells from the marrow inoculum. Monoclonal antibodies with specificity for cell surface antigens expressed by human T cells have been used in Dr. Ronald Gress' laboratory to deplete normal T cells from allogeneic marrow and to remove malignant T cells from autologous marrow. A clinical protocol has been developed to assess the feasibility of utilizing these marrows in the treatment of aggressive T cell malignancies in combination with marrow ablative chemotherapy and radiotherapy.

### Structure, Regulation and Function of Genes Involved in the Immune Response

Studies carried out in Dr. Dinah Singer's laboratory demonstrated that the swine class I MHC multigene family contains only seven members, and as such is the smallest known class I family. Recent studies have been aimed at the characterization of the structure, expression, and function of this family. Physical mapping has established that all of the genes are linked and resident within 500 kb. Determination of the DNA sequences of five members of the family have shown that the sequence organization of the class I SLA genes is similar to that of other class I genes in other species. Despite the small size of the family, three sub-groups of genes can be discerned based on their sequence homologies. Analysis of class I cDNA clones has established the exon organization of the genes, as well as their expression. Only four class I genes appear to be expressed in vivo. The three sub-groups defined by DNA sequence also display discrete patterns of expression. Thus, the genes of one



monoclonal antibody to enhance the engraftment of allogeneic marrow, these results indicate that it may be beneficial to time the injection of allogeneic marrow so that it is present during the period of T cell activation induced by the antibody in order to provide the possible benefit of factors which stimulate hematopoiesis.

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genes appear to be expressed *in vivo*. The three sub-groups defined by DNA sequence also display discrete patterns of expression. Thus, the genes of one sub-group are expressed ubiquitously, although their quantitative levels vary. A second sub-group defines a differentiation antigen. A third sub-group does not appear to be expressed at all.

Transgenic mice containing isolated swine class I genes have been developed as a model for investigating the molecular basis for the differential patterns of MHC gene expression. For one gene, encoding a classical transplantation antigen, it has been demonstrated that the pattern of expression of the swine gene in the transgenic parallels that observed *in situ*. Transgenics containing three other class I gene constructs have been generated.

Dr. Dinah Singer's laboratory has also initiated studies to characterize DNA sequence elements and cognate trans acting factors associated with class I MHC genes. Although the molecular mechanisms by which the regulatory elements and their factor function is not known, the current data suggest a model in which the negative and positive regulatory elements each define an independent binding site.

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

#### **LABORATORY OF CELL BIOLOGY**

A major emphasis in the Laboratory of Cell Biology, directed by Dr. Lloyd Law, is the identification and characterization of tumor specific transplantation antigens. Dr. Law and his colleagues demonstrated that the 3-methylcholanthrene-induced murine fibrosarcoma, Meth A, displays at least three different tumor-specific transplantation antigens (TSTA). One of these is an 82-kDa cytosolic protein that is also present in nontransformed cells. Another is a 96-kDa glucose-regulated protein. The third TSTA is a mixture of two heat shock proteins, designated HSP84 and HSP86. HSP84 and HSP86 are coded for by separate, but related, genes. Full-length cDNA clones have been isolated and their nucleotide sequences determined. The HSP structural gene has been mapped to a location near the major histocompatibility complex (MHC) class I region on chromosome 17. The HSP84 gene was sequenced and comprises approximately 6 kb of DNA. It is interrupted by 10 intervening sequences. The promoter contains structural features similar to those found in certain housekeeping genes. The genomic sequence of the HSP84 gene from nontransformed parental strain shows no differences in the deduced amino acid sequence compared with Meth A. Six other members of the HSP84 family were characterized and found to be processed pseudogenes having 70-80% homology to HSP84 cDNA. The HSP86 family consists of a structural gene and two highly homologous processed pseudogenes. These genes were assigned to chromosomes 12, 11, and 3. The HSP86 pseudogenes apparently arose more recently than those of the HSP84 family. Comparison of the HSP86 cDNA from the parental and other strains of mice has been performed for nearly the complete molecule. No nucleotide changes that would lead to amino differences have been observed. The manner in which HSP84/86 acts as a TSTA therefore remains an enigma. The tumor-specific

## SUMMARY REPORT

LABORATORY OF GENETICS, DCBD, NCI

October 1, 1988 through September 30, 1989

The major focus of the work in the Laboratory of Genetics is on B cell tumor development in mice, with particular emphasis on the plasmacytoma system. In addition, there are projects on studying the structural basis of antibody-protein antigen interactions by Dr. Smith-Gill. Dr. Coon continues his work on searching for the specificity of receptors in olfactory epithelium.

### Plasmacytomagenesis

The BALB/c mouse is uniquely susceptible to plasmacytoma development following the intraperitoneal injection of pristane or other paraffin oils. We are attempting to identify genes that determine resistance (R) and susceptibility (S). Two genes have been tentatively identified through linkage association: Pctr-1 (chr 4) and Pctr-3 (chr 17). We have been unable to confirm Pctr-2. We have constructed a congenic strain BALB/c.D2-Fv-1 that carries Pctr-1. After 19 backcrosses this mouse develops only less than 20% plasmacytomas. We are attempting to localize Pctr-1 using a 3-factor cross but have not been able to find a marker closer than 30 cM. Much of the effort is directed to finding a closely linked gene.

Beverly Mock has continued to identify a plasmacytoma resistance gene that controls plasmacytoma development induced by a combination of pristane and RIM virus (the virus that contains a myc oncogene and v-Ha-ras). She has found that the BALB/c.D2-Dag mouse is as resistant as the DBA-2 parent to RIM/pristane induction. She has also shown the BALB/c.D2-Dag mouse carries an additional DBA/2 gene, MMTV-13, that is not linked to Ly-6 (chr 15). A C.D2 Ly-6 congenic is susceptible to both pristane and pristane/RIM induced plasmacytomagenesis. She has screened 25 markers for linkage to this R/S gene. Her laboratory has developed libraries of mouse chrs 4 and 15 and is developing new markers on chromosomes 4 and 15 for this purpose. Since BALB/c.D2-Dag is a congenic mouse, this is an important new direction.

We, in collaboration with Dr. Ulf Rapp's laboratory, have continued our studies on the role of the raf-1 oncogene in plasmacytomagenesis and have found that the J3 virus, which contains a mutant and defective raf gene and a v-myc gene does not transform plasma cells. However, when this virus is passaged, it acquires plasmacytomagenic activity by correcting the frameshift mutation in v-raf. This work opens up a new area because this virus undergoes further mutational change in acquiring plasmacytomagenic activity.

We have continued to search for genes that cooperate with c-myc in plasmacytomagenesis and still have not been able to implicate members of the ras gene family (K. Huppi). Myc is apparently activated indirectly by the reciprocal translocations of rept(6;15). In man the activation of myc is thought to be mediated by base substitution mutations in c-myc. We have attempted to find out if similar mutations occur in the mouse. This work has not progressed in the mouse because of the unavailability of the 5 prime UT region of the c-myc

gene. This region has now been cloned by J. Shaughnessy and K. Huppi. Further, the myc genes from plasmacytoma, ABPC-22, which has no translocation, have been cloned, and one allele contains an unidentified insertion. The evidence indicates there are base substitution mutations in myc in the rcpt(6;15) translocations.

In experiments with L. Byrd (graduate student) we have found that the BALB/cAnN nu/+ mice that have been conventionalized in our colony are susceptible to plasmacytoma induction by pristane. This result has indicated that the specific pathogen-free status of BALB/cAn mice inhibits plasmacytoma induction. We are attempting to determine the mechanism of immunological experiences with various infections in rendering mice susceptible.

Drs. Shacter and Janz have been working on the mechanism of pristane action in plasmacytomagenesis. Much of this work has been impeded at the biochemical level because it has not been possible to introduce pristane into cells. Drs. Janz and Shacter have found a way to solubilize pristane so that it can be taken into cells. This is accomplished by making inclusion complexes between beta-cyclodextrin and pristane. Interestingly, beta-cyclodextrin/pristane inclusion complexes are biologically active.

#### Myeloid Tumors

Linda Wolff has continued her work on the induction of monoblastic tumors by the injection of Moloney virus in pristane-conditioned mice. She has shown that the virus must be injected intravenously and, further, that these tumors can be prevented by treating mice with indomethacin. She has just shown that splenectomy can inhibit myeloid tumor development. Her current work is directed to studying the role of prostaglandins in this process.

The Moloney virus inserts into the c-myb oncogene in virtually all of the monoblastic tumors. During the past year Dr. Shen-Ong has focused on the characterization of the protein products of the normal c-myb (75 kd) and tumor-specific truncated myb (70 kd) in murine cells and have generated an antiserum that specifically recognizes the novel myb isoforms (85 kd) that are generated by an alternative splicing event. We have also identified human cDNA clones that encode different C-terminus truncated forms of myb. We would like to find out the function(s) of the different forms of myb and have made various retroviral myb constructs to address this issue.

Studies in Dr. Ruscetti's section have concentrated on understanding the mechanisms by which the spleen focus-forming virus abrogates the erythropoietin dependence of erythroid cells and causes erythroleukemia. Their data indicate that the transmembrane portion of the viral envelope glycoprotein is responsible for conferring factor-independence on erythroid cells and that the mechanism does not involve factor production or an increase in the number or affinity of membrane receptors. Dr. Ruscetti's section has also made progress in understanding the molecular basis for the resistance of certain strains of mice to erythroleukemia induced by Friend MuLV. Their data suggest that resistant mice carry a unique provirus that encodes an envelope glycoprotein that blocks the spread of mink cell focus-forming viruses in these mice. Mice carrying this provirus also have a greatly reduced susceptibility to thymomas induced by Moloney MuLV.

### Antibody Protein Interactions

In order to define the structural basis of molecular recognition in protein-protein interactions, we have analyzed in detail the antibody antigen interface of monoclonal antibodies specific for the defined protein lysozyme, focusing on two antibodies for which the X-ray structures of the Fab:lysozyme complexes have been determined (the second, HyHEL-10, within the past year) in collaboration with Dr. David Davies (LMB, DIR, NIDDK). The effects of specific amino acid residues on specificity have been examined by cloning, expressing, and performing site-directed mutagenesis on the rearranged HyHEL-10 genes, and by site directed mutagenesis of cloned and expressed lysozyme genes. These two mutagenic approaches have produced unexpected results indicating nonadditive and long-range interactions, and are raising provocative questions concerning the nature of specificity and its relation to the biology and regulation of the immune response.

### Studies on Olfactory Epithelium

Dr. Coon's studies on rat olfactory epithelium have resulted in one publication (PNAS) and one abstract (AChem). We are attempting to mine the vein provided by the SNIF cell lines and to produce new and possibly better ones. We are looking at changes in differentiation markers as SNIF-11 differentiates after shift to serum-free medium; new markers are VIMENTIN and intermediate neuro-filaments in differentiated SNIF-11.





A. Genetics of Susceptibility to Plasmacytoma Induction. (with B. Mock, K. Huppi)

We continue to develop BALB/cAn.DBA/2 congenic strains of mice to determine the number and location of genes of DBA/2 origin that reduce the incidence of plasmacytomas. Last year we had identified three resistance genes: Pctr-1 (distal chr 4 linked to Fv-1, ANF and Rep-2); Pctr-2 (chr 15 linked to Ly-6) and Pctr-3 (chr 17 linked to Qa-2). We have completed the testing of the C.D2-Fv-1 at N20 and found it to be even resistant than when last tested at N10.

The identification of Pctr-2 was based on the finding that BALB/c-DAG carries Ly-6 on chr 15. These mice were derived after 14 backcrosses in another laboratory and they proved to be partially resistant to plasmacytoma induction. B. Mock has recently discovered that our BALB/c-DAG is segregating the MMTV-13 gene on chr 4. In the meantime we backcrossed the Ly-6 gene from DAG onto BALB/cAn for two generations and tested the new congenic BALB/cAn.D2-Ly-6 and found that it was much more susceptible to plasmacytoma induction nearing levels seen in BALB/cAn. Thus the resistance previously noted for the DAG mouse was due to the MMTV-13 linked gene, in all probability Pctr-1. Since the DAG mouse was developed using antisera rather than DNA probes it may be possible that the Pctr-1 gene may be associated with cell surface markers and this may facilitate finding this gene.

Our work is continuing to fine structure map the location of the Pctr-1 gene on chromosome 4. We have been hampered by the lack of polymorphic markers in the 30 cM stretch between ANF and MMTV-13, but recently two possible candidate genes are being evaluated, Akp and jun. We will attempt to locate Pctr-1 between ANF and any one of these markers then derive recombinants and test for plasmacytoma susceptibility. This work progresses very slowly because of the extensive amount of time required for the breeding and to test for plasmacytoma susceptibility.

Plasmacytoma Induction in nu/nu Mice, Role of Environmental Conditions. (with Linda Byrd, graduate student)

In initial studies it was found that BALB/cAnN-nu/nu mice obtained from C. Reader and originally bred at NIH by C. Hansen were resistant to plasmacytoma induction. Surprisingly so were BALB/cAnN nu/+ mice. In both situations the mice were maintained in pathogen free conditions. The BALB/cAn nu/+ mice were conventionalized in our colony and then reevaluated for susceptibility and it was found that they were now susceptible to plasmacytoma induction. While this result did not resolve the role of T cells in plasmacytomagenesis it has clearly demonstrated that the conventional microenvironment with the stimulus of a microbial flora and various viral infections is essential for tuning the immune system. We are now generating the reverse experiment by deriving a pathogen free BALB/cAnPt mouse and the induction studies with this should be under way this summer.

## Rapid Induction of Plasmacytomas with Retroviruses and Pristane

Plasmacytomas can be induced rapidly in pristane conditioned BALB/cAn mice that are infected with various types of transforming retroviruses. Last year we described the powerful plasmacytomagenic action of RIM virus that contains E $\mu$ -Myc

and V-Ha-ras. This virus can induce plasmacytomas in between 80 to 100% of mice given near sub-plasmacytomagenic doses of pristane (i.e. 0.2ml). Most of the tumors arise between 40 and 80 days and secrete IgM paraproteins. Dr. Marcu is preparing a new set of oncogenes for testing in this system.

Three years ago in experiments with Ulf Rapp we found that the J3 virus which contained a deleted raf gene and an avian v-myc under the control of viral LTRs induced plasmacytomas in pristane conditioned mice. In contrast the J2 virus which contained a complete v-raf-1 and v-myc induced the formation of myeloid tumors. We were not able to explain this result but have continued to study this problem. We have now gone back and had all of the virus preparations made in Ulf Rapp's laboratory by J. Troppmair. Surprisingly true J3 is only weakly if at all plasmacytomagenic. The plasmacytomagenic variant of this virus used in the experiments described in the Science paper has been passaged in vitro. It was hypothesized that J3 mutated in vitro. The J3 variant called J3 has now been sequenced by Troppmair and found to have undergone a second deletion that has restored the reading frame of the remaining part of the raf gene which contains the minimal transforming unit of the raf ser/thr kinase. In addition we have found that Y7 which contains A-raf + v-myc is also highly transforming. The reason for failure of J2 to transform plasma cells may be due to powerful action of the raf gene in J2. J2 is known to produce different types of transformed colonies than J3 and to transcribe smaller amounts of its raf genes. We have found evidence of other types of plasmacytomagenic recombinant viruses containing only v-myc genes which are currently being studied with great interest.

The induction of plasmacytomas in pristane conditioned mice by genes that cooperate with v-myc or  $E\mu$ -myc presents a powerful tool for testing a great variety of oncogenes for their ability to cooperate with dysregulated myc genes. We are now attempting to construct as many viruses as possible to test which kinases are active and which are not. This should facilitate defining the signal transduction pathways that are essential for transforming plasma cells.

#### B. Biochemistry of Induction of Plasmacytomagenesis - E. Shacter

The work has been directed mainly at identifying the carcinogenic agents that cause pristane-induced plasmacytomagenesis in BALB/cAn mice and at determining the biological activity of pristane. We have followed two main lines of inquiry:

- 1) Study the possible involvement of pristane-elicited phagocytes in promoting plasmacytomagenesis. Inflammatory neutrophils and macrophages comprise the major cell type in the oil granuloma and in the free peritoneal space for several months following the injection of pristane. Our working hypothesis is that these phagocytes, which lies in close proximity to infiltrating plasma cells, generate free radicals that can cause potentially mutagenic DNA damage in neighboring cells.
- 2) Determine whether pristane may have some direct tumor-promoting effect on B lymphocytes. Pristane is a branched chain alkane for which no mammalian metabolic pathways or genotoxic activities are known. Still, it is possible that a low level of uptake and/or metabolism of pristane in normal B lymphocytes could alter the growth potential of the cells or could lead to formation of low levels of carcinogenic agents. In order to examine this possibility, studies were carried



out to test for metabolism of pristane in the oil granuloma and to determine whether pristane may have any direct effects on the growth and viability of B lymphocytes.

#### Major Findings:

1. We have established an in vitro model system to study formation and repair of DNA damage induced by pristane-elicited neutrophils (Shacter et al., 1988). Our finding that neutrophil-induced damage was repaired more slowly than H<sub>2</sub>O<sub>2</sub>-induced damage suggested that induction of damage by activated neutrophils may be complex in nature and that this may contribute to the putative mutagenic potential of these cells. To test this hypothesis, experiments were carried out to determine which products of the oxidative burst are responsible for causing DNA damage and to determine whether components of the extracellular environment may participate in the damage process. It was found that exogenous agents, notably histidine, other amino acids, and metals interact with reactive compounds secreted by neutrophils to enhance the level of damage induced in neighboring tumor cells. The results suggest that secondary reactions such as site-specific radical formation may mediate neutrophil-induced damage, and they reveal that although secretion of H<sub>2</sub>O<sub>2</sub> is central to the mechanism whereby neutrophils induce damage, this oxidant alone does not account for all of the DNA damaging capacity of these phagocytes (Shacter et al., submitted). Future experiments will involve measuring oxidative damage in the oil granuloma.

2. (a) Pristane is completely immiscible with aqueous solutions and hence cannot be brought in contact with cells under normal in vitro cell culture conditions. Together with Siegfried Janz, we prepared artificial vehicles (liposome vesicles,  $\beta$ -cyclodextrin inclusion complexes) to introduce pristane to cells. It was consistently found that pristane is cytotoxic to B lymphocytes at relatively low levels ( $\geq 100 \mu\text{M}$ ) but that it has a small growth promoting effect (H3-thymidine incorporation) at a narrow range of sub-toxic levels ( $\sim 25 \mu\text{M}$ ).

(b) In collaboration with Drs. T. Jones and H. Fales (NHLBI), we extracted oil granuloma and peritoneal lavage samples and carried out GC/MS analysis to look for metabolites of pristane. None were detected.

(c) In collaboration with Drs. A. Herrmann and R. Blumenthal (NCI), we have found that pristane enhances fusion of enveloped viruses with erythrocyte cell membranes.

The possible implications of these results for plasmacytomagenesis will be examined in future experiments.

#### C. The Genetic Control of Plasmacytomagenesis - B. Mock

##### I. Pristane-Induced (PCTs)

Our basic approach has been to generate a series of F2 and backcross progeny from susceptible (BALB/c) and resistant (DBA/2) strains of mice which have been typed for the formation of plasma cell tumors. DNAs will be made from susceptible individuals and subjected to Southern analysis in an attempt to obtain markers closely linked to the susceptibility/resistance loci.



So far, 500 (CxD) F2 progeny have been typed for susceptibility to pristane-induced PCTs. Only 6 progeny (equivalent to 12 meiotic events) were susceptible, indicating an incidence of PCT formation consistent with a 3 gene model of plasmacytoma susceptibility. DNAs have been isolated from the susceptible F2's and southern analysis has been performed for ca. 26 markers to examine the extent of cosegregation between the susceptible phenotype and BALB/c alleles for the markers. To date, the lowest recombination frequencies have occurred with Ifa on chr 4, d on chr 9 and Sparc on chr 11.

The significance of these linkage analyses have been hampered by the low number of susceptible F2 progeny. As a result, we have generated an additional 300 backcross progeny and are currently in the process of typing these mice for PCT susceptibility. This induction study should be completed by August 1990, at which time, additional mice can be included in the linkage analyses.

In conjunction with these studies, we have collected and analyzed a large panel of probes for restriction fragment length polymorphisms (RFLPs) upon hybridization with BALB/cAn and DBA/2 DNAs. Our goal is obtain a minimum of 3 equally spaced markers/chromosome to insure a high probability of linkage when used in segregation analyses.

Previous studies (by M. Potter) involving C.D2 bilineal congenic strains, have indicated the existence of three Pctr genes in the BALB/c-DBA/2 model system, as indicated by studies in C.D2 congenic mice. These genes have been identified as follows: Pctr-1 (linked to Fv-1 on chr 4), Pctr-2 (linked to Ly-6 (DAG) on chr 15) and Pctr-3 (linked to Qa-2 on chr 17).

Recent molecular analyses of C.D2-Dag mice have shown that they not only carry the DBA/2 allele of Ly-6 on chr 15, but that they also carry the DBA/2 allele for MMTV-13 on chr 4. These results raise the possibility that what has previously been referred to as Pctr-2 is in fact the same gene as Pctr-1.

The mounting evidence for Pctr-1 on chromosome 4 has lead us to generate a chromosome 4 specific library from a somatic cell hybrid known to carry mouse chromosome 4. We have purified approximately 70 random clones from this library and are in the process of mapping them in an effort to obtain markers most closely linked to the disease locus.

In addition, numerous attempts to find RFLPs between BALB/c and DBA/2 from hybridization with chromosome 17 markers have resulted in identifying only one marker (Qa-2). Similar to the approach outlined above, I have generated a chromosome 17 specific library from a somatic cell hybrid line and plan to begin screening this library for additional clones in the near future.

## II. Pristane & RIM-induced PCTs

We utilized the phenotypic differences between BALB/c and DBA/2 mice to tumor formation in examining the genetics of susceptibility to RIM-induced plasmacytoma-genesis. RIM is a retroviral construct containing an immunoglobulin enhancer and promoter driven c-myc gene and a Moloney MuLV-LTR driven V-Ha-ras gene. Over the course of 6 separate induction studies, by day 120, 92% (n=83) of the BALB/c and 10% (n=75) of the DBA/2 mice developed PCTs. In addition, 85% (n=58) of the CxD Fl mice developed tumors, indicating that susceptibility is dominant with this

induction scheme. Interestingly, 48% (n=244) of the backcross progeny between BALB/c and DBA/2 mice were susceptible to PCT formation, indicating that a single gene is likely to control the development of RIM-induced PCTs in BALB/cAn mice ( $\chi^2=0.51$ ,  $p=0.5$ , i.e. no significant deviation from 50% susceptible: 50% resistant expected for a single gene model). Linkage analysis involving 20 of these backcross progeny for RFLPs associated with PCT<sup>S</sup> and PCT<sup>R</sup> mice, have not revealed significant linkage to ca. 20 markers. An additional 180 DNAs have recently been included in our panel of progeny and will be subjected to extensive Southern analysis in an effort to identify the chromosomal location of this susceptibility locus. Analysis of three C.D2 congenic strains have indicated that the Fv-1 and Ly6 loci on chromosomes 4 and 15 are not involved in pristane and RIM-induced susceptibility. Interestingly, the C.D2-Dag congenic is relatively resistant to pristane and RIM-induced PCTs. We are currently pursuing a detailed genetic profile of these mice in an effort to define the extent of DBA/2 chromatin in this congenic.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
 Z01 CB 08727-12 LGN

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Organization and control of genetic material in plasmacytomas

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

P.I.:	J.F. Mushinski	Medical Director	LGN, NCI
	G.L.C. Shen-Ong	Senior Staff Fellow	LGN, NCI
	K. Huppi	Staff Fellow	LGN, NCI
	H. Mischak	Guest Researcher	LGN, NCI
	A. McDonald	IRTA	LGN, NCI

COOPERATING UNITS (if any)

U. Rapp, FCRC, Frederick, MD; H.C. Morse, III, LVD, NIAID; F.D. Finkelman, Dept. of Medicine, USUHS; R. Eisenman, Hutchinson Cancer Center, Seattle, WA; M. Smulson, Georgetown University, Washington, D.C.

LAB/BRANCH

Laboratory of Genetics

SECTION

Oncogene

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

9

PROFESSIONAL:

5

OTHER:

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.)

The overall goal of this research is to study the activation of particular genes in diseased and normal cells in order to understand which genes may play important roles in the development of malignancies, autoimmune diseases and normal differentiation. The immune system has been chosen as the central focus of this research, and we have concentrated on the expression of "oncogenes," especially myc, myb, ras, raf, abl, rel and bcl-2, as well as immunoglobulin and T cell receptor genes. To this end we have been studying tumors that are regularly induced in BALB/cAnN mice by intraperitoneal injections of alkane mineral oils, such as pristane. These tumors represent immortalized lines of B lymphocytes or myeloid cells at different stages of differentiation which can be studied to learn how the genes involved in myeloid and B-cell carcinogenesis are organized and regulated. The deregulated expression of the myc oncogene has been clearly shown to be essential to the induction of plasma cell tumors, but the biochemical mechanisms responsible for this deregulation are still being elucidated. It is likely that other genes must also be activated to complete the neoplastic transformation. The ras family of oncogenes is a likely candidate, but we now have evidence that mutations in these genes are uncommon in plasmacytomas and lymphosarcomas. The myb gene is activated in certain virus-induced myeloid malignancies by insertion of a virus within the coding region. The effects on the protein products of altered forms of c-myb gene are being identified with specific anti-myb antisera, and alternative splicing of an extra coding exon has been found in normal and malignant cells. The putative proto-oncogene, bcl-2, appears to be expressed in a differentiation stage-specific manner in several different hematopoietic systems. We are testing whether overexpression of bcl-2 in mice is sufficient to induce tumors, and whether it can complement c-myc in tumorigenesis. Proto-oncogenes that have protein kinase activity are also under study in retroviral expressions vectors for their ability to induce hematopoietic tumors in mice.



Major Findings:Gene expression (J.F. Mushinski)

a. Expression of oncogenes in normal and neoplastic B-lymphocytes. We found that expression of the nuclear oncogenes c-myc, c-myb and c-fos, was highest in tumors of pre-B lymphocytes and that it diminished in tumors of more mature B cells. The opposite was found for expression of Ha-ras. A unique pattern of expression was found for bcl-2 in that very early progenitor B cells (Pro B cells) such as HAFTL-1 and HAFTL-3 and very late plasmablasts and plasmacytomas contained no bcl-2 mRNA, while pre B and mature B cells did contain bcl-2 transcripts. HAFTL-1 and HAFTL-3 can acquire many characteristics of myeloid cells, and this phenotypic change is accompanied by the onset of expression of bcl-2. Model systems of other hematopoietic cells are being studied to see whether bcl-2 is frequently down regulated when these and other cell types are induced to differentiate. Mouse erythroleukemia cells differentiate into hemoglobinproducing cells following exposure to DMSO. This differentiation is accompanied by cessation of bcl-2 expression. Myeloid cell lines on the other hand sometimes increase and sometimes decrease expression of bcl-2 when differentiation is induced by various agents.

b. Increased v-myc expression can induce murine lymphoid and myeloid tumors. Constitutive expression of c-myc is a consistent finding in pristane-primed BALB/c plasmacytomas and is usually a consequence of chromosomal translocation involving the c-myc locus. The requirement for the translocation can be replaced by retroviruses that express v-myc or c-myc, which verifies the essential role myc expression plays in induction of such tumors. Myeloid tumors are also induced by myc retroviral constructs. Careful study of DNA from successive generations of several myeloid and B-lymphocytic tumors induced by J1, J2, J3 and J5 viruses which contain different combinations of v-myc and/or v-raf indicates that these two oncogenes act synergistically to induce tumors rapidly and occasionally multiple tumors at the same time. Viruses expressing only v-myc generally induce only myeloid tumors. Viruses expressing myc plus raf commonly induce B lymphoid neoplasms, which suggests a synergistic complementation of two proto-oncogenes in lymphocytic neoplasia. We (with U. Rapp) are searching for mutant viruses in these cell lines. We (with H. Mischak) are constructing new retroviruses which express both myc and protein kinase genes in order to study other possible oncogene synergies in B-cell neoplasia.

c. Mutations and Control of c-myc expression. Deregulation of myc expression appears to be one of the key elements in plasmacytomagenesis, and up regulation of c-myc expression is well known to accompany stimulation of cell growth. Thus control of c-myc expression in normal and neoplastic cells continues to be a major interest. Sequence studies of the c-myc gene in two plasmacytomas, TEPC1165 and TEPC2027, which contain unusually high levels of c-myc mRNA, have revealed hitherto undetected point mutations in myc intron 1 in these two tumors, so we (with K. Huppi) are now widening our DNA sequencing study of c-myc to look for mutations in this gene in other plasma cell tumors, including some with no karyologic abnormalities.

Virus-mediated or transgenically-induced myc overexpression appears to be able to bypass some of the resistance to pristane-induced plasmacytomagenesis in non-BALB/c mice. Thus the myc locus of BALB/c or the proteins that interact with it must be important in conferring on this strain its unique susceptibility to

plasmacytoma induction. Thus we (with A. McDonald) have also undertaken to clone and sequence the exons and flanking regions of the normal BALB/c, BALB/Jax and DBA/2 genes. We also will characterize and compare the nuclear proteins that interact with structural and regulatory regions of this gene in the susceptible and resistant strains.

d. Non-homologous recombination generates IgD-secreting plasma cells. Hybridomas (generated by F.D. Finkelman) secrete abundant antibodies of the IgD subtype. We have discovered that all of such cell lines have deleted the C $\mu$  gene. Thus despite the absence of a switch  $\delta$  region, heavy chain switching can occur normally using a recombinational mechanism. We are determining the precise locations of the recombination in all these cell lines by cloning and sequencing.

#### Study of myb protooncogene (Grace Shen-Ong)

We have previously identified a group of Abelson virus-induced myeloid tumors (ABMLs) which carries a disrupted myb allele as a result of integration of the helper Moloney murine leukemia virus (M-MuLV). cDNA cloning and sequencing analyses showed that 71 amino acids of the 5' coding sequences were removed in mRNAs transcribed from the disrupted allele. The open reading frame of these cDNAs predict the synthesis of a smaller tumor-specific myb protein. One of the ABML cDNA clones contains an additional in-frame internal myb exon, termed E6A, that is spliced out of the most abundant form of c-myb mRNA. The presence of the E6A sequences should cause the synthesis a myb isoform that is 10 kilodaltons larger than the major form of myb protein.

In order to test these predictions, we (with Robert Eisenman) used myb-specific antibodies to immunoprecipitate all the c-myb proteins from normal mouse thymocytes and tumor cells. The predicted major 75kd c-myb and an 85kd c-myb protein species containing E6A sequences were identified in cells that have normal c-myb loci. In ABML cells with a c-myb allele which was truncated at the 5' end by proviral insertion, two truncated myb proteins [a 70kd (lacking E6A) and a 80kd species (containing E6A)] were identified. We have developed rabbit antisera against bacterially expressed E6A sequences which recognize only the E6A-containing myb isoforms. All the different forms of myb were found to be localized in the cell nuclei, to have rapid turnover rates (~20min), and to be expressed throughout the cell cycle.

In order to determine whether the above-mentioned details of mouse myb expression of E6A can be generalized to other species, we (with J.F. Mushinski) have isolated E6A-containing myb clones from human cDNA libraries. RNase mapping studies revealed that this alternative form of myb is present in all human cells that express myb. Comparison of the murine and the human E6A sequences shows that this exon is similar but somewhat divergent between the two species.

We have also found another common alternative splicing event that can result in the production of a 3' truncated form of human c-myb protein. Elucidation of the regulation of expression of the structurally distinct myb protein isoforms should contribute to our understanding of the involvement of this gene in hematopoietic differentiation and tumorigenesis.

Genetic Variation in B lymphocytic tumors (Konrad Huppi).

Several lines of evidence indicate that more than one genes are involved in the transformation of mouse plasmacytomas (as outlined above). Since chromosomal translocation which relocates the c-myc to an Ig locus is a fundamental characteristic of most mouse plasma cell tumors, c-myc must be one of these genes. Several oncogenes are known to be capable of cooperating with c-myc in the transformation process in vitro. Among these are the ras family genes which have been shown to be mutated frequently in exon 1 (codons 12 & 13) or exon 2 (codons 59-61) in many human or rodent solid tumors. We have surveyed the mouse plasmacytomas for evidence of somatic mutation of Kras, Hras or Nras genes by Southern hybridization restriction analysis or by direct cloning and sequencing of genomic or PCR amplified fragments. The results of this survey reveal that ras mutations in mouse B cell tumors appear to be rare. However, a mutated Kras gene has been identified in the plasmacytoma TEPC 1174. This mutation is located at residue 23 and involves a Leucine to Arginine substitution. An NIH/3T3 assay of DNA from mouse plasmacytomas (with V. Notario, Georgetown University) reveals significant transformation capability for TEPC 1174. We conclude that the mutation at codon 23 of Kras results in a novel transforming phenotype.

One of the major mechanisms in the acquisition of mutations is thought to be loss of fidelity in DNA repair. We have determined that two regions, proximal chromosome 1 and distal chromosome 4, carry genes which are involved in efficient DNA repair in the mouse. These studies have led us to examine genes involved in the DNA repair process which map to these regions. Human poly (ADP-ribose) polymerase (ADPRP) is an automodification enzyme with DNA binding properties which is stimulated by DNA strand breaks. We (with M. Smulson) have cloned and sequenced cDNA and genomic DNAs for the mouse ADPRP structural gene. We have found close genetic association between the mouse ADPRP locus on mouse chromosome 1 and the generalized lymphadenopathy disease or gld locus associated with mouse autoimmunity. We (with D. Klinman, NINCDS) are investigating whether the gld mutation is, in fact, a mutation in ADPRP associated with defective DNA repair.

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<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b>		<b>PROJECT NUMBER</b>
<b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		Z01 CB 05553-19 LGN
<b>PERIOD COVERED</b>		
October 1, 1988 to September 30, 1989		
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b>		
Immunoglobulin structure and diversity. Characterization of cell membrane proteins		
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</b>		
P.I.: Stuart Rudikoff	Microbiologist	LGN, NCI
W. Davidson	Visiting Scientist	LGN, NCI
J. Hyde	Staff	LGN, NCI
D. Hilbert	Guest Researcher	LGN, NCI
<b>COOPERATING UNITS (if any)</b>		
<b>LAB/BRANCH</b>		
Laboratory of Genetics		
<b>SECTION</b>		
<b>INSTITUTE AND LOCATION</b>		
NCI, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b>	<b>PROFESSIONAL:</b>	<b>OTHER:</b>
6	4	2
<b>CHECK APPROPRIATE BOX(ES)</b>		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		B
<input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)</b>		
<p>1. IL-6 has been identified as a cytokine involved in a number of biological activities including the differentiation of lymphocytes. We have demonstrated, using IL-6 specific antisera, that this factor is absolutely required for primary, antigen specific B-cell responses while secondary responses are IL-6 independent. Analysis of a series of Th-1 type T-helper clones revealed a qualitative difference in their ability to provide help in terms of antibody secretion. Clones producing IL-6 were able to support both primary and secondary responses while clones lacking IL-6 could support only secondary responses. These clones could help primary responses if IL-6 was added exogenously indicating that this lymphokine provides a critical functional difference in terms of the ability of different T-cell subpopulations to support antibody responses.</p> <p>2. Gene inactivation studies based on homologous recombination in embryonal stem (ES) cells have been initiated. ES cells have been obtained and are currently being introduced into recipient blastocysts to generate chimeric animals as a test of the pluri-potency of the cell line. Two genes, the immunoglobulin C<math>\kappa</math> constant region and the Ia alpha chain, have been selected for initial studies. Both have been inactivated by cloning the neomycin resistance gene into an expressed exon. Inactivated genes are currently being introduced into ES cells following which the cells will be tested for homologous recombination events. Assuming positive results are obtained, ES cells with inactivated genes will be used to attempt the production of corresponding chimeric animals.</p>		

## A. Cell Growth and Differentiation

In the preceding annual report, we described the initiation of experiments to study genes controlling cell growth and differentiation. The major effort of the laboratory has continued in this direction and individual projects are summarized below.

1. We have previously characterized a macrophage derived factor, IL-6, required for in vitro growth of a number of murine plasmacytomas. The role of this cytokine in normal B-cell and thymocyte differentiation has been investigated during the course of this year. Initial observations that IL-6 is absolutely required for primary antigen specific B-cell responses, but secondary B-cell responses are IL-6 independent have been confirmed.

The source of IL-6 in these responses has been analyzed by characterization of a series of antigen specific T helper clones. T helper clones are classified as to type based on the particular lymphokines produced. A survey of a large group of such clones specific for the antigen used in these studies (the hemagglutinin of influenza) revealed that all were of the Th-1 type. However, among these, differences were observed in the ability to secrete IL-6 permitting a further subdivision of this category of helper clones. In functional assays employing representative members, it was found that clones which secreted IL-6 were capable of providing help for both primary and secondary responses. However, clones which did not secrete IL-6 could help only secondary responses. Addition of exogenous IL-6 restored the ability of these clones to help primary responses indicating that IL-6 was the critical molecule providing the functional difference between the two helper clones. Additional studies are currently underway to determine whether IL-6 has a similar role in T independent responses.

One of the major aims of this project has been the production of monoclonal antibodies to the active site of IL-6. The reagents are required to perform in vivo studies to elucidate mechanisms of plasmacytoma generation and growth as well as potential IL-6 involvement in wasting syndromes resulting from tumor therapy regimens with such agents as TNF. Antibodies will be used in attempts to inhibit both plasmacytoma induction and transplantation as we believe IL-6 is a critical molecule in both processes. These same antibodies will be used in tumor rejection models to determine whether modulation of observed high IL-6 levels proves therapeutically beneficial. To date, a number of IL-6 specific monoclonal antibodies have been produced by immunization with purified or recombinant material. None of these inhibits biological activity and are thus unsuitable for the proposed experiments. As an alternative approach, peptides have been synthesized corresponding to the region we believe to include the active site. Polyclonal antibodies are being made to these peptides to determine if the antibodies produced will inhibit biological activity. If a positive result is obtained, these peptides will be used to further attempt the preparation of site specific monoclonal antibodies for use in the above described studies.

2. A second system being developed to assess function of genes believed to be involved in regulation employs the use of homologous recombination in embryonic stem (ES) cells to inactivate particular genes being studied. It has been demonstrated that pluri-potential ES cells can be manipulated in cul-

ture and then re-introduced into blastocysts to give rise to normal offspring. The approach being taken is to inactivate a given gene by cloning a disrupting sequence into one or the exons and to then replace the normal counterpart by homologous recombination.

To facilitate experimentation, we have arbitrarily divided the system into two parts with the first being the embryo and animal work and the second the cell and molecular biology. In animal experiments, we have been able to isolate and re-implant 8-cell stage blastocysts with resulting live births. Currently, isolated blastocysts are being injected with ES cells obtained from Oliver Smithies to determine whether we can actually generate germ line chimeras. On the cell and molecular biology side, two genes, the immunoglobulin kappa chain constant region (Ck) and the Ia alpha chain, have been selected for study. Constructs have been made of both in which the gene is inactivated by cloning the neomycin resistance gene into one of the exons. ES cells have been successfully grown in culture and conditions established for electroporation. The constructs are now being introduced into these cells following which the cells will be analyzed for homologous recombination. Successful completion of this stage will permit us to then proceed with the introduction of ES cells containing inactivated genes into blastocysts to attempt to generate animals with inactivated genes for biological studies.

3. A third approach to identifying novel genes involved in cell growth and differentiation consists of the construction of subtractive cDNA libraries between cells representing different ontological stages of the same lineage. Attempts to induce differentiation of clonal B-cell lines have been largely unsuccessful, although in one case a pre-B line has been induced to differentiate into mature macrophages (see below under Wendy Davidson). We have therefore resorted to the construction of cDNA libraries from unrelated B-cell lines. The major effort in this project over the last year has been to technically optimize the experimental system in an effort to obtain lower frequency messages. This goal has been achieved and the first subtraction using a pre-B and mature B cell as the parental types has been performed. Based on the results from this library, additional cell lines representing intermediate stages of development will be included to generate a more complete picture of gene expression in this lineage.

#### Publications

Davidson WF, Pierce JH, Rudikoff S, Morse HC III. Relationships between B cell and myeloid differentiation: studies with a B lymphocyte progenitor line, HAFTL-1. *J Exp Med* 1988;168:389-407.

Singer DS, Hare J, Golding H, Flaherty L, Rudikoff S. Identification and characterization of a new sub-family of class I genes in the H-2 complex of the mouse. *Immunogenetics* 1988;28:13-21.

Osbourne BA, Golde TE, Schwartz RL, Rudikoff S. Evolution of the IgA heavy chain gene in the genus *Mus*. *Genetics* 1988;119:925-31.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05552-20 LGN

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Mammalian cellular genetics and cell culture

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

P.I.: H.G. Coon	Research Biologist	LGN, NCI
R. Swerdlow	Staff Fellow	LGN, NCI
A. DeGrassi	Visiting Scientist	LGN, NCI
A. Mancini	Guest Researcher	LGN, NCI

## COOPERATING UNITS (if any)

Dr. Doron Lancet, Weizmann Institute, Tel Aviv, Israel (Aug '89-July '90 NICHD, LDP).

## LAB/BRANCH

Laboratory of Genetics

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

3.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

## SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

It is the purpose of this project to analyze and develop new and difficult systems for cell culture. We are now pursuing intensively a single cell system: culture of cells from the neonatal rat olfactory epithelium (OLFE). Our progress with the OLFE has been sufficiently exciting during the past year that it has merited our full attention. Using complex media and substrates we have succeeded in culturing several cell types from the OLFE. The mixed, mass cultures of these cells provide an appropriate conditioned medium that has permitted the isolation of >20 clonal cell strains from 3rd to 6th passage cultures. We have shown that several of our cloned cell lines have sensitive (submicromolar) and selective (different response patterns in each line) odorant-dependent second messenger responses (both cAMP and Ca<sup>++</sup>). This fact, coupled with our demonstration that these same cell strains are positive for neuron-specific enolase, GAP43, as well as carnosine and carnosine synthetase, now establish that we have right cell type in culture. We have continued the hybridoma screen using OLFE as antigen and fluorescent anti-mouse IgG staining of frozen sections of OLFE for selection. Development of this system would make available the first mammalian neuroblast to neuron cell culture system and provide a means to study the growth and differentiation dichotomy common to all blast cell systems. It is hoped that basic issues in olfactory sensory physiology can be explored with this system.

Major Findings:

Much of the time in the early part of the year was spent in getting data and figures together for our paper describing the SNIF cell lines. Since that was submitted, we have tried to follow 3 lines of approach to advancing the project: 1) Dr. R. Swerdlow is characterizing the appearance and disappearance of known markers in SNIF-11 during "step-down" as they change from rapid cell division in protein rich medium (4506) to quiescent and apparently differentiating neuroblasts in plain, protein free medium (DMEM). His goal is to identify a developmental series showing developmental regulation as this nearly unique cell system differentiates in vitro. 2) Dr. Coon and Drs. A. Mancini and A. DeGrassi are trying to develop fresh isolates of the SNIF cell series for biochemical investigation of the characteristics of newly isolated cells in vitro as opposed to the long term cell lines that we isolated in the spring of 1986. 3) We are currently starting experiments designed to find out whether evidences of pluripotentiality seen in our cell lines in vitro can be confirmed in vitro. This project is being done in collaboration with Dr. Scott Freeman (from W. French Anderson's lab, NHLBI MH) and Drs. P.P.C Graziadei and G.A. Monti Graziadei, Florida State University, Tallahassee. This report consists of a brief description of the findings to date for these three projects.

1) The finding that blastemetic cells (specifically the murine neuroblastoma C-1300) may cease dividing and proceed to commence differentiation when "stepped-down" from serum containing medium to serum free medium is not new (see citation in our PNAS paper below). The method has been exploited in PC-12 cells as well, except that nerve growth factor has to be included in the serum free medium. Using SNIF-11, the cell strain that shows step-down most dramatically, we have found neuron specific enolase, Gap 43 (a marker of growing axon terminals), carnosine (a putative neural transmitter in the olfactory system) and carnosine synthetase activity. We have not found: olfactory marker protein, light neurofilaments (68 kD), synaptophysin or tyrosine hydroxylase, the latter two perhaps not surprisingly. Medium neurofilaments have been shown by immunohistochemistry and until "western blot analysis" is complete must be considered inconclusive. There is also preliminary evidence that the fos gene product is present. n-myc (found in human neuroblastomata) will be sought, as well as a range of newly available olfactory specific transduction molecules: the  $G_{olf}$  recently described by Drs. Randy Reed at Hopkins and Dr. Doron Lancet at the Weizmann, as well as an olfactory specific adenylyl cyclase ( $AC_{olf}$ ). These investigators have agreed to supply probes and some antibodies. Dr. Lancet will be spending the next year at NIH and is also interested in collaborating in characterizing the olfactory cytochrome p450 and uridine diphosphate glucuronyl transferase that he has been working on recently.

The finding that NGF has an effect on stepped-down SNIF cells (longer and more elaborated processes) is interesting and possibly important. Dr. Swerdlow has been characterizing the effects of NGF and other factors that we have found in the past year to effect the differentiation process in SNIF-11. The other factors are IL-6 and bFGF. Either IL-6 or bFGF are sufficient when added in nanogram quantities to cause cell division to proceed in otherwise protein free DMEM. Dr. DeGrassi, who found the IL-6 response, is particularly interested in further exploring the fascinating connection between factors and markers in the immune system and the nervous system using our olfactory neuroblast cell lines.

2) The effort to develop new cell strains of the SNIF cell type has proven difficult and filled with bad luck and disappointments. At this writing we are encouraged by a group of clones growing in 50% conditioned medium. The long process of isolating these and their subsequent characterization over the next year is a necessary, time consuming and tedious part of this whole project.

3) In a past report we alluded to the fact that some of our cloned cell lines from the rat olfactory epithelium were able to produce cartilage, muscle, fat cells, glial fibrillary acidic protein (a glial cell marker) as well as the neuron marker, neuron specific enolase. This surprising result made the cultures, which we thought were derived from the stem cell population of "basal" cells in the olfactory epithelium, appear to be like F9 or other embryonal carcinoma cells in culture or like the result of treating 10T1/2 cells with 5-azacytidine pluripotent cells. The olfactory stem cells and perhaps other stem cell populations could be much more multipotential than heretofore imagined. The suggestion is that the olfactory stem cells make whatever their local environment specifies. Perhaps nose cells would make retinal cells in the eye or gut cells in the crypts? Perhaps the strategy of the nervous system is to produce first a "generic neuron" and then to further differentiate it according to its location in the CNS as a whole. Such a notion is also inherent in work with grafts in the CNS performed over the years by the Graziadeis. Hence, we will try to put these notions to a critical test by labelling the cultured cells or the in vivo "basal" cells with a defective retrovirus carrying beta-galactosidase. The progeny of a successfully infected cell will be identifiable uniquely by histochemistry which will reveal cells in sections carrying this nonmammalian enzyme. The Graziadeis have many years of experience with such grafts and their location by stereotaxis. Dr. Coon has the cells and experience with making grafts from cell cultures for evaluation of differentiated function (e.g., thyroid and parathyroid) as well as ample experience with the somatic cell genetic procedures. The constructs will come from Dr. Freeman.

Publications:

Coon HG, Curcio F, Sakaguchi K, Brandt ML, Swerdlow RD. Cell cultures of neuroblasts from rat olfactory epithelium that show odorant responses. Proc Natl Acad Sci USA 1989;86:1703-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
201 CB 08950-07 LGN

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Molecular recognition by protein effector molecules

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

P.I.:	Sandra Smith-Gill	Microbiologist	LGN, NCI
	C.R. Mainhart	Microbiologist	LGN, NCI
	T.B. Lavoie	Biologist	LGN, NCI
	C.P. Mallett	Staff Fellow	LGN, NCI
	A. Newman	Student Volunteer	LGN, NCI

COOPERATING UNITS (if any)

A.B. Hartman, Dept. of Biologics, Walter Reed; D. Davies, LMB, NIADKD; A.C. Wilson, J.F. Kirsch, University of Cal. Berkeley; S. Sheriff, Squibb Institute for Medical Research

LAB/BRANCH

SECTION

Laboratory of Genetics

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

4.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

Protein-protein interactions underlying molecular recognition are studied using monoclonal antibodies directed against the model protein antigen, lysozyme  $\zeta$  (HEL). The X-ray structures of two Fab-HEL complexes for 2 of these antibodies (HH5 and HH10) have been refined, and we are now studying in detail the molecular mechanisms underlying specificity and affinity in protein-protein interactions. Site-specific mutants of an expressed cDNA clone of HEL have shown that the same substitution in adjacent contact residues can have dramatically different effects on antibody (HH5) binding, results that are directly relevant to understanding "escape" mutants arising from antigenic drift. Site-directed mutants of expressed HH10 genes have shown that: (1) somatic mutation can alter fine specificity as well as affinity; (2) long-range interactions can significantly influence, molecular recognition, (3) antibodies can be multispecific; (4) antigen-specific antibodies may recognize an antigen multivalently. The development of specificity for HEL from an apparently multispecific available antibody repertoire is currently being examined in detail utilizing large panels of hybridoma antibodies. The HEL-induced response is characterized by the absence of IgM HEL-reactive precursor cells are recruited into the IgG compartment very early. It is also possible that multispecific B-cells may be precursors for antigen-specific clones.



Project Description:

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

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

HyHEL-5 is a highly specific mAb in which electrostatic interactions contribute a significant portion of the binding energy. We originally were able to identify Arg68<sub>HEL</sub> as critical residue in the epitope recognized by HyHEL-5. The recently refined X-ray structure of the HyHEL-5 Fab:HEL complex confirmed this prediction, and revealed that both Arg68<sub>HEL</sub> and Arg45<sub>HEL</sub> form salt links with Glu residues from the antibody heavy chain. Site specific mutants of HEL were produced in the laboratories of A.C Wilson and J.F. Kirsch (Department of Biochemistry, University of California, Berkeley). Binding studies with these mutant lysozymes indicated a significant quantitative difference between the energetic contribution of the 2 Arg<sub>HEL</sub> residues to the antibody:HEL interaction. The R68<sub>HEL</sub> → K substitution lowered affinity by almost 10<sup>4</sup>, while the R45<sub>HEL</sub> → K substitution lowered affinity only by a factor of 10.

The results with HyHEL-5 are directly relevant to understanding viral "escape" mutants, for R → K substitutions in two adjacent contact residues have dramatically different effects on antibody binding; a substitution such as R68<sub>HEL</sub> → K which reduces antibody affinity by approximately 10<sup>4</sup> might be sufficient to escape neutralization, while the smaller quantitative effect of the R45<sub>HEL</sub> → K substitution probably would not. We are continuing detailed analyses of these interactions utilizing several interrelated approaches: (i) free energy calculations (being done by J. Novotny and colleagues, Squibb Institute for Medical Research); (ii) free energy measurements by microcalorimetry, in collaboration with D. Davies and colleagues (NIDDKD); (iii) structural (Xray crystallography) studies on mutant complexes, in collaboration with D. Davies and S. Scheriff (Squibb); (iv) site specific mutagenesis of HyHEL5 rearranged genes, which we are in the process of cloning, in collaboration with A.B. Hartman (Walter Reed Army Medical Center); (v) comparative structure-function studies with other antibodies recognizing the same epitope, including X-ray crystallography. A. Newman and C. Mallett have recently partially characterized several other mAbs from BALB/c mice immunized with HEL that appear to be sensitive to the R68<sub>HEL</sub> → K replacement found in bobwhite quail lysozyme (i.e., low binding to bobwhite quail but not to other quail lysozymes) that also appear to be of lower affinity than HyHEL-5.

The serilogically-defined epitopes recognized by the structurally related mAbs HyHEL-8 and -10 are very similar, and the affinities of the two antibodies are comparable. However, while HyHEL-10 is very sensitive to a variety of amino acid substitutions in HEL, HyHEL-8 is relatively insensitive to the same substitutions. In addition, they are structurally related to the IgAK DNP-binding myeloma protein, XRPC-25. T. Lavoie has cloned and expressed the genomic rearranged heavy and light chain genes for HyHEL-10 and has produced a series of oligonucleotide-directed mutants. Analysis of these mutant antibodies has yielded the following conclusions, some unexpected and novel: (i) somatic mutation can dramatically alter antibody affinity; (ii) somatic mutation can alter fine specificity (i.e., ability to cross-react with altered antigen) without significantly altering affinity; (iii) point mutations in antibody residues that do not contact antigen can significantly affect antigen recognition, i.e., long range interactions can significantly influence function; (iv) antibodies can be multispecific, i.e., can specifically recognize more than one structurally unrelated antigen; (v) an antigen-specific antibody may recognize the antigen multivalently, i.e., may recognize more than one epitope on a protein antigen. An understanding of the structural bases of these binding properties is essential to the design of recombinant antibodies or of vaccines which would elicit natural antibodies with specificity properties that are minimally subject to the loss of neutralizing activity due to mutation of the target.

In addition, a comparison of serological data with the HyHEL-10 X-ray structure has presented several apparent dilemmas; serological mapping was less accurate in predicting the epitope recognized by the antibody HyHEL-10 than it was for HyHEL-5. In particular, non-overlapping (by competition assays) antibodies appear to recognize the same amino acid residues, and non-contact HEL residues appear to significantly influence binding. These results again point to the significance of long-range interactions.

We are continuing analysis of these interactions using several approaches: (i) production of additional site-specific mutants of both antibody and antigen; (ii) structural studies on HyHEL-8, as well as several other antibodies with related specificities (in collaboration with S. Sheriff); (iii) modeling and structural studies designed to identify the "second site" seen by antibodies which recognize HEL multivalently. C. Mallett has recently characterized several new mAbs which recognize epitopes near or overlapping that of HyHEL-10 which will be very valuable in analyzing the indicated possible long-range interactions.

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

In order to document the genesis of specificity from an apparently poly-reactive preimmune repertoire, C. Mallett has produced a panel of over 400 BALB/c hybridomas representing the course of the immune response to HEL. An unexpected finding was that in the HEL-immunized hybridomas, not a single IgM mAb was monospecific for HEL at any point of the immune response. Conversely, there were IgM antibodies that were specific for other antigens, including "rheumatoid factors" specific for IgG. In addition, polyreactive IgM mAbs were derived from all of the immunization schedules and in most instances the frequency was similar to IgM hybridomas derived from blasts or naturally activated spleen cells. HEL-specific IgG antibodies, on the other hand, were derived from all

but one of the immunization schedules. Furthermore; a high frequency of polyreactive IgG mAbs which react with apparently unrelated antigens were recovered from HEL-immunized mice. An additional unexpected finding was that a significant number of HEL-specific IgG antibodies appeared to recognize HEL as a multivalent antigen.

Analysis of a panel of over 500 hybridomas, derived from polyclonally activated and unstimulated (naturally activated) spleen cells by P. Rousseau, revealed that HEL-specific precursors do exist in the available preimmune repertoire. Although monospecific precursor cells are rare, monospecific IgM precursors as well as multireactive HEL-binding blast-derived hybridomas were found at frequencies comparable to other protein antigens in the panel. Over half of the hybridomas from polyclonally activated adult splenic B cells produced IgM antibodies that were antigen reactive, and of these over half reacted with 5 or more structurally dissimilar complex antigens, including HEL. Naturally activated IgM antibodies include a population which closely reflects the specificity characteristics of the available repertoire, including HEL-reactive antibodies. Thus, the absence of HEL-reactive primary response IgM antibodies cannot be attributed to an absence of available precursors, and most likely reflects an early recruitment of HELreactive clones into the peripheral B cell pool. Our results also suggest the possibility that multireactive as well as monospecific HEL-reactive B cells are recruited into the IgG peripheral B cell pool very early in the course of the immune response, and that polyreactive B cells may serve as precursors for some HEL-specific IgG antibodies. We are currently undertaking detailed structural and functional analysis of selected antibodies from these panels.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

201 CB 08951-07 LGN

PERIOD COVERED

October 1, 1988 to September 30, 1989

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)

PI: S.K. Ruscetti	Senior Investigator	LGN, NCI
A. Saxena	Visiting Fellow	LGN, NCI
A. Chakraborti	Visiting Fellow	LGN, NCI

COOPERATING UNITS (if any)

Dr. W.D. Hankins, Armed Forces Radiobiology Research Institute, Bethesda, MD

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

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

Studies on the acute erythroleukemia-inducing spleen focus-forming virus (SFFV) have concentrated on understanding how this virus abrogates the erythropoietin (Epo) requirement of erythroid cells. The unique envelope glycoprotein encoded by SFFV has been shown to be responsible for this biological effect and we now show using molecular recombinants between variants of SFFV that sequences present in the transmembrane domain of the viral envelope glycoprotein are essential for rendering the cells Epo-independent. Studies designed to understand the mechanism by which SFFV alters the Epo requirements of erythroid cells should be aided by our recent observation that an Epo-dependent erythroleukemia cell line can be rendered Epo-independent by infection with SFFV. As previously shown with spleen cells from SFFV-infected mice, the induction of factor-independent in these cells by SFFV does not appear to involve a classical autocrine mechanism involving Epo. The factor-independent cells, however, eventually acquire the ability to produce a unique growth factor that may contribute to their growth. Using the Epo-dependent erythroleukemia cell line and its SFFV-infected counterpart, we can find no evidence of induction of protein tyrosine phosphorylation by either Epo or SFFV.

Studies on the genetics of susceptibility to erythroleukemia induced by Friend MuLV continue to concentrate on the Rmcf locus, which encodes or regulates the expression of an MCF-related envelope glycoprotein. Our recent data indicate that this protein may be encoded by a modified polytropic provirus that is present in Rmcf<sup>F</sup> mice but not Rmcf<sup>S</sup> mice. In other studies, we show that the Rmcf gene also effects susceptibility to the thymic lymphoma induced by Moloney MuLV.

Major Findings:

I. The transmembrane domain of the envelope protein of the polycythemia-inducing strain of SFFV determines Epo-independent growth.

Erythroid cells from mice infected with the polycythemia-inducing strain of SFFV (SFFV<sub>p</sub>), unlike normal erythroid cells, can proliferate and differentiate in the apparent absence of the erythroid hormone erythropoietin (Epo). The unique envelope glycoprotein encoded by SFFV has been shown to be responsible for this biological effect. Using env-gene recombinants between variants of SFFV, we have shown that a 120 bp region at the 3' end of the env gene of SFFV<sub>p</sub> must be present in order for the virus to render cells Epo-independent. Since the 120 bp fragment encodes the entire p15E-related hydrophobic transmembrane region of the envelope glycoprotein, this suggests that minor differences in the membrane anchoring portion of the protein might be critical in determining Epo responsiveness. Our data also indicate that the transmembrane region of the SFFV<sub>p</sub> envelope protein is not just serving an ancillary function related to subcellular localization as previously suggested, since a correlation no longer exists between the level of expression of the envelope glycoprotein at the cell surface and the Epo dependence of these cells. These results indicate that the transmembrane region of the SFFV<sub>p</sub> envelope glycoprotein is playing a critical role in mediating the effects of the virus on erythroid cells.

II. SFFV<sub>p</sub> induces factor-independent growth and tumorigenicity in an Epo-dependent erythroleukemia cell line.

To further examine the ability of SFFV<sub>p</sub> to abrogate Epo dependence, we introduced the virus in complex with various helper viruses into an Epo-dependent erythroleukemia cell line, HCD-57, derived from a mouse infected at birth with Friend MuLV. Selection of the SFFV-infected cells in medium lacking Epo efficiently and reproducibly gave rise to lines which were both factor-independent (FI) and tumorigenic. FI lines could not be recovered from uninfected HCD-57 cells or from cells infected with helper virus alone, indicating that SFFV contributed to their factor-independent phenotype. The SFFV-infected FI lines express high levels of the SFFV envelope glycoprotein and release infectious SFFV. As previously shown using spleen cells from SFFV<sub>p</sub>-infected mice, the induction of factor-independence by SFFV<sub>p</sub> in HCD-57 cells does not appear to involve a classical autocrine mechanism involving Epo, since the cells do not secrete Epo and do not contain detectable levels of Epo mRNA, even when tested in a sensitive assay exploiting the polymerase chain reaction. In addition, anti-Epo serum has no effect on their proliferation. When the cells were examined for their ability to bind Epo, the SFFV-infected cells bound 5-fold less Epo than the parental line, indicating that SFFV products may either block the Epo receptors in these cells or down-modulate it. This result is in contrast to studies using spleen cells from SFFV<sub>p</sub>-infected mice, which showed no difference from uninfected erythroid cells in their ability to bind Epo. FI lines can also be derived, at lower efficiency, from HCD-57 cells infected with the anemia-inducing strain of SFFV but not from cells infected with an Abelson MuLV stock that clearly abrogates IL3-dependent cells from their factor dependence. Preliminary results with a variety of other oncogenic viruses, including those containing v-ras, v-src, v-myc, v-erbB, v-src and v-myb/ets, indicate that SFFV is the only virus tested so far that can abrogate HCD-57 cells from their Epo-dependence.

Preliminary results with SFFV-infected FI HCD-57 cells suggest that at some point after virus infection they begin producing a factor other than Epo which can support the growth of the parental cells. This factor is not secreted into the medium and its effects can only be demonstrated when irradiated SFFV-infected HCD-57 cells are cocultivated with the parental cells. This factor may be related to a unique factor recently shown to be produced by Rauscher murine erythroleukemia cells (Migliaccio et al., Blood, in press). In both cases, data are consistent with the hypothesis that production of this growth factor is the result of SFFV integrating into a specific site in mouse DNA and is not related to expression of the SFFV envelope glycoprotein in these cells. Thus, transformation of the Epo-dependent HCD-57 cells by SFFV may involve 2 steps, analogous to the multiple stages of disease that exist in SFFV-infected mice. The first step would be the abrogation of Epo-dependence by the expression of the viral envelope glycoprotein and interference with a post-receptor step in the Epo signal transduction pathway. The second step would be the integration of SFFV in a specific site in the DNA and activation of a gene that produces a unique growth factor that can stimulate the growth of the HCD-57 cells by an autocrine mechanism.

III. Neither Epo nor SFFV induce tyrosine phosphorylation of proteins in erythroid cells.

Since our data indicates that the SFFV envelope glycoprotein is effecting a post-receptor step in the Epo signal transduction pathway, understanding the mechanisms by which the Epo receptor transmits its mitogenic signal may provide insights into how SFFV-infected cells proliferate abnormally. Activation of protein tyrosine phosphorylation is an integral response in many different mitogenic systems. Although neither the Epo receptor nor the SFFV envelope glycoprotein contain tyrosine kinase domains, it does not rule out the possibility that these proteins activate protein tyrosine phosphorylation in their signal transduction pathways. To examine the effects of Epo and SFFV on protein tyrosine phosphorylation, we used the Epo-dependent murine erythroleukemia HCD-57 cell line along with its SFFV-infected counterpart as well as normal erythroid cells from the spleens of phenylhydrazine-treated mice along with spleen cells from SFFV-infected mice. Extracts of these cells before and after stimulation with Epo were analyzed by immunoblotting with various sources of antibodies specific for phosphotyrosine. Using this technique, we can find no evidence for Epo- or SFFV-stimulated tyrosine phosphorylation of specific proteins in erythroid cells. We are currently examining these cells for stimulation of serine and threonine phosphorylation of target proteins.

IV. DNA from mice carrying the  $Rmcf^R$  locus contain a unique provirus not present in the DNA from  $Rmcf^S$  mice.

Previous studies utilizing a series of BALB/c mice congenic for various DBA/2 genes have shown that DBA/2 mice carry a gene on chromosome 5, at or near the  $Rmcf^R$  locus, that plays a major role in resistance to early F-MuLV-induced erythroleukemia. One can detect in resistant mice an 80K glycoprotein related to the envelope glycoprotein of MCF viruses that is thought either to be encoded for by this locus or whose expression is influenced by the locus. Susceptible mice carrying the  $Rmcf^S$  locus do not express detectable levels of the 80K MCF virus-related envelope glycoprotein. In order to determine if the  $Rmcf$  gene



is a viral structural gene or a gene regulating the expression of the structural gene, we examined DNA from susceptible BALB/c mice (Rmcf<sup>S</sup>) and resistant BALB/c mice that were congenic for the Rmcf<sup>F</sup> locus. Using synthetic oligonucleotide probes specific for polytropic or modified polytropic viruses, we were able to detect a provirus in the DNA of the Rmcf<sup>F</sup> congenic mice that was missing in Rmcf<sup>S</sup> mice. These results indicate that the Rmcf<sup>F</sup> gene is a viral structural gene related to the envelope gene of a modified polytropic virus. We are currently attempting to clone this provirus using cDNA prepared from the Rmcf<sup>F</sup> congenic mouse.

#### V. The Rmcf gene effects susceptibility of mice to thymic lymphoma induced by Moloney MuLV.

Although our data clearly indicates that the Rmcf<sup>F</sup> gene renders mice resistant to the early erythroleukemia induced by Friend MuLV, it was not known whether or not this gene effected the diseases induced by other retroviruses. In order to examine this, we infected various strains of mice with Moloney MuLV and examined them for the development of thymic lymphoma. Our data indicates that mice carrying the Rmcf<sup>F</sup> locus, while not completely resistant to thymic lymphoma, developed a significantly lower incidence of disease (5-10-fold) when compared with mice carrying the Rmcf<sup>S</sup> locus. Since it had previously been shown that susceptible NIH Swiss (Rmcf<sup>S</sup>) mice, after infection with Moloney MuLV, developed a preleukemic state characterized by generalized hematopoietic hyperplasia, we were interested in whether or not the Rmcf<sup>F</sup> gene would inhibit this preleukemic state. Rmcf<sup>F</sup> and Rmcf<sup>S</sup> mice were infected with M-MuLV and at various times after infection their hematopoietic tissues were examined for the number of CFU-C and CFU-E as well as for the replication of MCF viruses. Our data indicate that mice carrying the Rmcf<sup>F</sup> gene differ significantly from mice carrying the Rmcf<sup>S</sup> gene in the expression of MCF viruses in their hematopoietic tissues but do not differ significantly in the development of a generalized hematopoietic hyperplasia. These results suggest (1) that MCF virus replication is not necessary for the development of generalized hematopoietic hyperplasia in M-MuLV-infected mice and (2) that development of a generalized hematopoietic hyperplasia is not sufficient for the development of thymic lymphoma. These results suggest that MCF virus replication is important in the development of M-MuLV-induced thymic lymphoma and that its effect may be related to a role of Moloney MCF viruses in insertional mutagenesis rather than in generating a preleukemic state in the hematopoietic tissues.

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## Major Findings

I. Studies on the preleukemic phase of Moloney MuLV induced promonocytic tumors: splenectomy reduces tumor incidence.

The fact that intravenous and not intraperitoneal inoculation of Moloney MuLV is effective in inducing tumors which subsequently arise in the peritoneal cavity suggests that there is a preleukemic stage of tumor development of these tumors, splenectomies were performed at various times following virus inoculation. It was observed that splenectomy inhibited tumor formation by 73% when carried out 4 weeks following i.v. injection of virus. When performed before virus injection, or 2 weeks or 6 weeks after virus injection inhibition of tumor formation ranged from 24 to 46%. A theory to explain these results is that the target cells for transformation by the virus can be found in the spleen, but not exclusively in this area since tumors can be formed at a reduced incidence in splenectomized mice. Interestingly, it was found that pristane-primed mice have an increased number of granulocyte-macrophage colony stimulating factor (GM-CSF)-responsive cells (CFU-C) in both their spleens and livers when compared to untreated mice.

II. Inhibition of promonocytic tumors with indomethacin.

A. Effect of indomethacin on established transplantable tumors. Previously, the tumor inhibitory effect of indomethacin, a nonsteroid anti-inflammatory drug, was tested and shown to dramatically reduce development of promonocytic tumors. When indomethacin was put in the drinking water of BALB/c mice, starting prior to treatment with pristane and inoculation of M-MuLV, tumor incidence was reduced from 50% to 0%. In order to determine whether the indomethacin treatment was effective against outgrowth of established malignant cells, transplantable tumors were tested for their ability to grow in the peritoneal cavity of pristane-treated mice that were receiving indomethacin in their drinking water. Tumor latency in mice receiving indomethacin was only slightly shorter than in mice not receiving indomethacin. Furthermore, only a small reduction in overall incidence could be detected when experiments were carried out using as few as 100 tumor cells per mouse. It was concluded from these and our previous studies that the ability of indomethacin to inhibit primary tumor formation following inoculation of MuLV lies in its ability to block outgrowth of cells of early stages of tumor cell development. Further support for this contention comes from the fact that recent tumor induction experiments utilizing virus have shown that if indomethacin is started two weeks after virus inoculation instead of before virus inoculation, the inhibitory effect of indomethacin is abrogated.

B. Production of prostaglandin E<sub>2</sub> by cells in the peritoneal cavity of pristane primed mice and by promonocytic tumor cells. Indomethacin is known to block the formation of prostaglandins, prostacyclin, thromboxanes and intracellular oxygen radicals through its inhibition of cyclooxygenase an enzyme that converts arachidonic acid into these products. Several models to explain the tumor inhibitory effects of indomethacin can be drawn depending upon which products(s) of arachidonic acid metabolism are important in tumor promotion. Since prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is known to be produced by macrophages and cells from the ascites fluid of pristane primed are known to contain macrophages, it was reasoned that this might be an important substance involved in tumor growth enhancement. Furthermore, the promonocytic tumor cells had been shown to have characteristics of macrophages

and therefore could be producers of PGE as well. Using radioimmune assays for PGE<sub>2</sub> it has been possible to show that cells from the ascites fluid of pristane-primed mice and both primary tumor cells derived in vivo as well as tumor cell lines grown in vitro can produce high levels of PGE. In vivo derived tumor cells do not have to be stimulated to produce PGE, whereas tumor cells that have been propagated in vitro have to be stimulated with lipopolysaccharide before PGE production is observed. Both types of tumor cells show a complete reduction in PGE production when treated with indomethacin in vitro.

G. Reversal of inhibitory effects of indomethacin using prostaglandin E<sub>2</sub>. To test whether tumor inhibitory effects of indomethacin have been working through the ability of indomethacin to block PGE<sub>2</sub> specifically, mice revealing pristane and virus and indomethacin in their drinking water were also given two intraperitoneal injections per day of dimethyl-PGE<sub>2</sub> (33ug/kg) in PBS. In contrast to control mice receiving injections of PBS alone, mice receiving PGE<sub>2</sub> developed tumors starting at 75 days post virus inoculations. These results indicate that the PGE<sub>2</sub> is specifically promoting tumor growth and that indomethacin can abrogate its effects.

### III. Genes in Moloney MuLV specifically related to promonocytic tumor development

A. Ability of other replication competent viruses to induce promonocytic tumors in pristane-treated mice. Some preliminary experiments in our laboratory suggested to us that amphotropic virus (Ampho 4070) is also capable of inducing promonocytic tumors under similar conditions whereas Friend MuLV could not produce the disease. We have subsequently determined that Ampho 4070, a virus which is usually considered to be of low leukemogenicity, when injected intravenously in pristane-primed DBA/2 mice is very pathogenic: it can produce tumors starting at about 3 months and the incidence of tumor formation is greater than 50%.

B. Studies on genetically modified Moloney MuLV. Results of in vivo testing of reciprocal recombinants between Friend and Moloney MuLV indicated that there are structural gene(s) that are present in Moloney MuLV, but not Friend MuLV, that are required for induction of promonocytic tumors. Furthermore, our results indicate that there are sequences in the U3 region of Moloney MuLV which are crucial for its pathogenicity to myeloid cells because interruption of these sequences (through insertion of nonretroviral sequences) blocks specific induction of the promonocytic tumors, but does not interfere with general replication competency in the mouse or the ability of the virus to induce lymphoid tumors.

#### Publication:

Wolff L, Mushinski JF, Shen-Ong GLC, Morse III HC. A chronic inflammatory response: its role in supporting the development of c-myc and c-myb related promonocytic and monocytic tumors in BALB/c mice. 1988 J Immunol 141:681-89.

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## SUMMARY STATEMENT

### LABORATORY OF MOLECULAR BIOLOGY

DCBD, NCI

OCTOBER 1, 1988 TO SEPTEMBER 30, 1989

The Laboratory of Molecular Biology uses genetic and molecular biological approaches to study gene activity and cell behavior and to develop new approaches to the diagnosis and treatment of cancer, AIDS and other human diseases.

#### Regulation of Gene Activity

##### I. Pastan

The EGF receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To study regulation of the EGFR gene, the promoter region was isolated, sequenced and found to be very GC-rich but did not contain a TATA sequence. It does contain many transcription factor binding sites including four Sp1 sites and sites for at least three other factors. One of these factors is a 120 kilodalton protein termed ETF that binds to three sets of GC-rich sequences and stimulates EGF receptor transcription in cell-free extracts. When these sequences are present in front of a  $\beta$ -actin or SV40 promoter that contains TATA sequences, transcription is not affected by ETF. To isolate transcription factors that bind to the GC-rich region, a labeled fragment was used to screen a cDNA expression library made from the RNA of A431 cells, and a cDNA clone encoding a 95 kilodalton protein was isolated. The function of this protein which may be a transcriptional repressor is under investigation.

#### Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells:

##### M. M. Gottesman and I. Pastan

Resistance to multiple drugs is a major impediment to the successful chemotherapy of human cancers. One mechanism of multidrug-resistance (MDR) is the expression of a 170,000 dalton energy-dependent drug efflux pump (P-glycoprotein) which confers resistance to colchicine, adriamycin, vincristine, vinblastine, puromycin, and actinomycin D. ATP-dependent transport of vinblastine has been demonstrated using membrane vesicles from MDR cells. This transport is inhibited by vincristine, daunomycin, actinomycin D and colchicine as well as verapamil, quinidine and other drugs which reverse multidrug-resistance. Expression vectors into which the MDR1 cDNA is cloned confer the complete MDR phenotype on drug-sensitive cells, as does a retrovirus carrying the MDR1 gene. When introduced into dog kidney epithelial (MDCK) cells, the MDR1 cDNA product is expressed on the apical surface of the epithelia, and allows transport of drugs such as vinblastine, daunomycin, actinomycin D and verapamil. A chimeric P-glycoprotein, in which a functional adenosine deaminase (ADA) molecule is attached to the C-terminus, has been constructed. Introduction of this cDNA into cells results in drug resistance

and ADA activity simultaneously. Transgenic mice carrying the human MDR1 gene expressed in bone marrow were produced and shown to be resistant to daunomycin-induced leukopenia. These mice will be useful in the testing of new drugs designed to overcome multidrug resistance. Expression of MDR1 RNA and P-glycoprotein normally occurs in kidney, liver, colon, and adrenal and in cancers derived from these tissues which are intrinsically resistant to chemotherapy as well as several other tumors. Acquired drug resistance in leukemia, lymphoma, neuroblastoma, rhabdomyosarcoma, pheochromocytoma, breast and ovarian cancer is associated with increased MDR1 RNA levels.

### Immunotoxin and Oncotoxin Therapy of Cancer Cells

I. Pastan, D. FitzGerald, and M. C. Willingham

*Pseudomonas* exotoxin (PE) or genetically modified forms of PE have been attached to monoclonal antibodies (mAbs) or growth factors to create cell-specific cytotoxic agents. Native PE attached to mAb OVB3 is cytotoxic to ovarian cancer cells, inhibits the growth of ovarian cancer in mice, and is being currently tested in a Phase I trial in women with ovarian cancer. Mutant PE molecules in which domain I has been replaced by TGF $\alpha$ , IL2, IL4, or IL6 have been created by the technique of gene fusion and the chimeric proteins produced in *E. coli*. TGF $\alpha$ -PE40 kills cancer cells with EGF receptors. IL2-PE40 kills certain types of leukemia cells as well as activated T cells involved in allograft rejection. IL6-PE40 is cytotoxic to some myeloma cells. CD4 has also been fused to PE40 to create CD4-PE40, a chimeric toxin which kills cells infected with HIV. Such molecules may be useful in the elimination of tumor cells or other cells such as HIV infected cells responsible for several human diseases.

### Development of Immunotoxins for Cancer

D. J. FitzGerald

To study the structure-function relationships of *Pseudomonas* exotoxin (PE) a series of monoclonal antibodies were selected and eight characterized and shown to react with either domains II, Ib, or III of PE. Two reacted with native PE; the other six reacted with cryptic epitopes which became exposed when PE was immobilized on nitrocellulose or denatured with urea and DTT. The hydrophobic properties of PE were explored. Full length PE or domains I, II, and III were each shown to bind more detergent at pH 4.5 than at pH 7.0. PE has an amino acid sequence resembling a consensus cleavage site for HIV protease. While PE was not degraded by this protease, PE40 was susceptible and was cleaved between residues 389-390.

Because large quantities of LysPE40 are required for immunotoxin production, a method was developed to produce it in gram quantities. By controlling pH, aeration and adding nutrients, cells expressing LysPE40 were grown to very high density and LysPE40 was purified from the medium using Q Sepharose, QMA, Mono S and gel filtration chromatography. LysPE40 immunotoxins were made with the following antibodies: HB21 (anti-TFR), antiTac (anti-IL2R), OVB3 (anti-ovarian Ca), anti-CD3, anti-CD5, and anti-CD33. OVB3-LysPE40 is poorly cytotoxic, primarily because the OVB3 antigen is not well internalized. To increase the rate of endocytosis, and thereby enhance cytotoxic activity, transferrin was chemically coupled to OVB3-LysPE40 to produce a conjugate that is about ten-fold more active than OVB3-LysPE40.

## Morphologic Mechanisms of Organelle Function and Transformation in Cultured Cells

M. C. Willingham

Neoplastic transformation produces many changes in cell function, some of which have been studied using morphologic techniques. Morphologic methods were developed to isolate monoclonal antibodies to tumor-specific epitopes for use in therapy of human cancers of ovary, colon, breast and stomach. One of these, OVB3, is currently in clinical trials as part of an immunotoxin for the treatment of ovarian cancer. Two new antibodies, OVM3 and OVM4, are being evaluated. New strategies for screening of monoclonal antibodies and for immunization are being developed. Antibodies C219 directed at the human multidrug transporter were found to cross-react with a myosin isoform. The ability of the multidrug transporter to pump protons out of cells was studied by measuring the alkalization of the cytosol using fluorescence photometry and microinjection of pH-sensitive dyes.

## Functions, Structure, and Regulation of Receptors for Cell Adhesion Proteins

K. M. Yamada

Receptors for key extracellular proteins such as fibronectin, collagen, and laminin are thought to mediate adherence, migration, and invasion by tumor cells, as well as analogous processes by certain normal cells. Membrane glycoproteins that bind collagen or fibronectin are being characterized. One collagen-binding protein is a transformation-sensitive heat-shock protein. A second binding protein termed VLA-2 may be central to normal and tumor cell penetration of collagenous matrices. Fibronectin receptors were shown to be altered in quantity and/or distribution in vivo and in vitro. Levels are increased in Rous sarcoma-virus induced tumors, and its membrane localization is altered in a variety of tumor cells. The structure of human fibronectin receptors was determined by a variety of electron microscopic approaches; it has an unusual shape with two long stalk-like legs. Subunit-specific monoclonal antibodies elucidated fibronectin receptor function in a variety of processes. The beta subunit, shared by other receptors, appears required for normal cell attachment to fibronectin, laminin, and collagen. The alpha subunit provides specificity. This fibronectin receptor was required for organization of an extracellular fibronectin matrix, specialized adhesive contacts, and thick intracellular microfilament bundles. It also restrains migration of normal fibroblasts as part of its function in maintaining the stationary cell phenotype. It thus appears to play a central role in regulating normal cell adhesion and migration.

## Structure and Roles of Transformation-sensitive Cell Surface Glycoproteins

K. M. Yamada

Fibronectin plays a crucial roles in cell adhesion, migration, invasion, and metastasis. Four key sequences in fibronectin involved in its recognition by cells are being characterized. One site containing the Gly-Arg-Gly-Asp-Ser sequence is required for epithelial as well as fibroblastic cell adhesion to fibronectin. A synthetic peptide containing this sequence inhibits experimental metastasis; its rapid rate of renal clearance was decreased by coupling it to a carrier. A second, synergistic cell-interaction sequence was defined by recombinant DNA mutagenesis. This site is required for cell adhesion, transmembrane effects on the cytoskeleton, and cell migration; its crucial



minimal sequence is being defined. A third site, present in some fibronectin molecules due to alternative splicing, was recognized in a cell-type specific fashion by neural crest cells and derivatives. Vitronectin, another major cell adhesion protein, was found to be regulated by an extracellular molecule termed PG-M, a novel proteoglycan that modulates adhesion via its chondroitin sulfate side chains.

### Development and Uses of Eukaryotic Vectors

B. H. Howard

Studies are being focused primarily on growth regulation in mammalian cells with investigation of growth inhibitory activity emphasized within this framework. Several new results of particular interest have been obtained. i) Growth regulatory properties of 7SL RNA and Alu elements have been shown to reside in two short DNA sequence motifs: the B block of the RNA polymerase III promoter and an undecanucleotide, GAGGCNGAGGC, which is homologous to papovavirus origins of DNA replication. ii) Screening of an Okayama-Berg cDNA expression library has led to identification of several clones differentially expressed in serum-starved and senescent human skin fibroblasts. iii) A new procedure for construction of full length cDNA expression vector libraries has been developed.

### Synthesis and Function of a Transformation-Dependent Secreted Lysosomal Protease

M. M. Gottesman

Cultured mouse fibroblasts which are malignantly transformed or treated with TPA or growth factors such as PDGF synthesize and secrete the 39,000 Mr precursor to cathepsin L in large amounts. Overproduction of cloned cathepsin L in a nontransformed cell results in secretion of this lysosomal enzyme. Cathepsin L is an active acid protease whether or not it is glycosylated. Secreted procathepsin L can bind to the mannose 6-phosphate receptor of many cells and be endocytosed and processed intracellularly. In antigen presenting cells, procathepsin L uptake reduces the efficiency of antigen presentation, thereby interfering with immune response. Transformation, TPA and PDGF stimulate transcription. A functional procathepsin L gene was cloned from mouse cells and the 5' flanking region which acts as a promoter in CAT constructions sequenced. Regulated transcription requires sequences in the first two exons and introns. A human procathepsin L cDNA has also been cloned and sequenced. Recombinant human procathepsin L produced in bacteria is enzymatically active. A deletion analysis of procathepsin L produced in bacteria indicates that both the "pro" and the carboxy-terminal part of the protein are needed for full activity.

### Genetic and Biochemical Analysis of Cell Behavior

M. M. Gottesman

The manner in which cyclic AMP regulates cell growth and gene expression in CHO cells and the function of EGF receptors in KB cells is being studied. The mechanism of cAMP action on CHO cells has been studied by isolating CHO mutants resistant to growth inhibition by cAMP. These mutants have defective cAMP dependent protein kinases with either altered regulatory (RI) or catalytic (C) subunits. DNA from cells carrying dominant cAMP-resistant defects transfers the cAMP-resistant phenotype to sensitive cells. Cosmid clones carrying the

CHO RI subunit from cAMP resistant cells have been isolated and the exon-intron structure of most of the CHO RI subunit gene has been determined. A full-length cDNA for CHO RI has been isolated and sequenced. The yeast phosphodiesterase has also been expressed in CHO cells. Expression of the PDE gene results in insensitivity to cAMP. With I. Pastan, KB cells selected for resistance to a conjugate of Pseudomonas toxin with EGF have been shown to have reduced levels of EGF receptors due to a decrease in EGF receptor mRNA. Many of these mutants are dominant in gene transfer experiments, and some show reduced growth in response to EGF.

### The Transgenic Mouse as a Model System to Study Gene Function and Regulation G. T. Merlino

Two important aspects of cancer: the ability of malignant cells to escape destruction by chemotherapy, and the role of oncogenes and growth factors in the development of cancer are being investigated. Transgenic mice which contain a human cDNA (MDR1) encoding a multidrug transporter (P-glycoprotein) capable of conferring multidrug resistance in cell culture have been generated. Expression of the human MDR1 gene was detected in the normally drug-sensitive bone marrow of several lines of transgenic mice. The transgenic bone marrow became resistant to the cytotoxic effects of the drug daunomycin. Since bone marrow suppression is a major drawback to the use of chemotherapy, these transgenic mice will be used as a model to determine if higher doses of drugs can cure previously unresponsive cancers. The epidermal growth factor (EGF) receptor is the cellular homolog of the avian erythroblastosis virus erbB oncogene product. To elucidate the role of the EGF receptor and its ligands in growth control and transformation, mice bearing either the human EGF receptor gene or the human TGF $\alpha$  gene were produced. In both cases the transgene was expressed in a wide variety of mouse tissues. Studies are underway to determine the effects of inappropriate expression on the developing mouse.

### Mechanisms of Thyroid Hormone Action in Animal Cells S.-y. Cheng

A cDNA clone encoding a human cytosolic thyroid hormone binding protein (CTHBP) has been isolated. The sequence of CTHBP was found to be homologous to the subunit of rat pyruvate kinase, subtype M2 (PKM2). CTHBP is a monomer that has 10-15% of the enzymatic activity of tetrameric PKM2. The tetrameric PKM2 does not bind 3,3',5-triiodo-L-thyronine ( $T_3$ ). Binding of CTHBP to  $T_3$  or its analogs resulted in the inhibition of pyruvate kinase activity. The apparent  $K_i$ 's of  $T_3$ , L-thyroxine, and D- $T_3$  are 30 nM, 100 nM, and 2  $\mu$ M, respectively which correlates with the thermogenic effects reported for  $T_3$  and its analogs. Since PK is a key enzyme in regulating cellular ADP, ATP, and pyruvate, CTHBP may be involved in mediating some of the cellular metabolic effects induced by thyroid hormones.

Using a T7 expression system in E. coli, large amounts of the human placental c-erbA protein (hc-erbA- $\beta$ ) were expressed and purified to apparent homogeneity. The purified hc-erbA- $\beta$  binds to  $T_3$  with a  $K_a = 2.8 \times 10^9 M^{-1}$  with  $n = 0.3$  mole/mole. It binds to  $T_3$  analogs with the same order of affinities as the  $T_3$  analogs bind to the  $T_3$  nuclear receptor present in tissues or cultured cells. Furthermore, the purified hc-erbA- $\beta$  binds to the  $T_3$  response element of the rat

growth hormone gene in a hormone-independent manner. The availability of large amounts of active hc-*er*BA- $\beta$  should lead to an understanding of the mechanism of transcriptional regulation by T<sub>3</sub>.

### Regulation of the gal Operon of Escherichia coli

S. Adhya

Gal repressor protein negatively regulates the expression of the gal operon of E. coli. Repression requires binding of repressor to two spatially separated operator sites, O<sub>E</sub> and O<sub>I</sub>. Previously it was shown that occupation of the two operators sites by repressor is not sufficient to achieve normal repression, and it was proposed that an interaction between two operator bound dimeric repressors, giving rise to a DNA loop, is essential for repression. The following experimental results strongly support this model of repression.

- i) Repressor dimers bound to O<sub>E</sub> and O<sub>I</sub> associate generating a DNA loop as studied by electron microscopy.
- ii) Repressor mutant protein was isolated which binds to O<sub>E</sub> and O<sub>I</sub>, but does not tetramerize. This mutant protein when bound to DNA does not form a DNA loop as studied by electron microscopy.
- iii) The mutant repressor fails to repress the gal operon.

It was also discovered that in a cell devoid of Gal repressor, a derepressed gal operon can be further induced two- to three-fold by the addition of the inducer, D-fucose. In vivo operator titration assays suggest that the gal operon is subjected to a new negative control under conditions when the classical Gal repressor is removed from the cell.

### Genetic Regulatory Mechanisms in Escherichia coli and Its Bacteriophage

S. Adhya and S. Garges

Cyclic AMP binds to its receptor protein CRP, and induces a conformational change (allosteric transition) in the protein. The cyclic AMP-CRP complex, but not free CRP, binds at or near many E. coli promoters in a sequence-specific way and either represses or activates transcription. Several classes of mutations in the crp gene were isolated and characterized, which identify the amino acids participating in the cyclic AMP-induced allosteric shift in the protein. On the basis of these results and the three-dimensional structure of the protein, it is proposed that cyclic AMP brings about a hinge reorientation to release the DNA-binding F  $\alpha$ -helices; alignment between two subunits; and an adjustment between the position of the two domains in each subunit. Extensive amino acid substitutions at some of these positions further supports the idea that a hinge movement involving specific interhelical polar-polar interactions and Van der Waals repulsions between side chains of defined amino acids are an essential part of the activation of CRP. Mutants have been obtained that are in an allosterically frozen conformation that is uninducible by cAMP. These mutations impart a defect in DNA binding and/or in transcription activation, and these data suggest that a cAMP-induced allosteric change is necessary not only for DNA binding but also for CRP-RNA polymerase interaction. The CRP-RNA polymerase interaction when bound to DNA is also being studied. Results suggest that a specific DNA distance between the two bound proteins is required for productive interaction. It is likely that there is a complicated arrangement between the two sites, in terms of DNA sequence and distance.

## Bacterial Functions Involved in Cell Growth Control

S. Gottesman

The role that protein degradation plays in regulating cell growth control is being investigated through the study of the proteases which carry out ATP-dependent proteolysis in *E. coli*. The Lon protease has previously been shown to degrade RcsA, a positive regulator of capsule synthesis, and SulA, a cell division inhibitor. The analysis of capsule synthesis is being continued. RcsC, a membrane protein, probably functions to sense environmental signals and transmits this information to the positive regulatory protein RcsB. RcsA may act directly on the regulated genes, or indirectly by affecting RcsB-RcsC interactions. The Clp protease, a second major ATP-dependent protease, has an ATP-binding subunit (ClpA) which shares homology with a family of proteins in both eukaryotic and prokaryotic cells. The protease subunit of the Clp protease (ClpP) contains a serine active site, and has only limited activity in the absence of ClpA. Clp is induced anaerobically; one target of Clp degradation is excess ClpA subunit. A third energy-dependent protease, the Alp protease, is capable of substituting for Lon under some conditions.

## DNA Replication in vitro

S. Wickner

The molecular mechanisms involved in the initiation of replication are being investigated, as well as how DNA replication is regulated. An in vitro replication system is being used that replicates plasmid DNA carrying the bacteriophage P1 origin of replication and many host functions that participate in the replication reaction have been determined, including dnaA, dnaB, dnaC and dnaG proteins, RNA polymerase, DNA gyrase and DNA polymerase III. Three of the E. coli heat shock proteins, dnaJ, dnaK, and grpE proteins are also required. How these proteins assemble on the initiation site and how they function in initiation has been determined. Using P1, it was shown that the P1 initiator protein, RepA, binds to the origin and control region simultaneously, causing the intervening DNA to loop as determined by electron microscopy. This looping probably inhibits initiation just as Dr. Adhya has shown looping in the gal operon inhibits gene transcription. Replication in vitro is restored when seven or more of the nine RepA binding sites are deleted from the control region or when wild-type RepA protein is substituted with RepA protein isolated from a repA mutant that has an increased copy number in vivo.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08000-19 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Regulation of Gene Activity

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

PI:	I. Pastan	Chief, Laboratory of Molecular Biology	NCI
	G. Merlino	Expert	LMB, NCI
Other:	A. Johnson	Staff Fellow	LMB, NCI
	R. Kageyama	Visiting Fellow	LMB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Molecular Biology

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

2.5

## OTHER:

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 EGF receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To study regulation of the EGFR gene, the promoter region was isolated and shown to contain many transcription factor binding sites including four Sp1 sites and sites for at least three other factors. One of these factors is a 120 kilodalton protein termed ETF that binds to GC-rich sequences found at positions -230 to -250. When these sequences are present in front of a  $\beta$ -actin or SV40 promoter that contains TATA sequences, transcription is no longer affected by ETF. To isolate transcription factors that bind to the GC-rich region, a labeled fragment was used to screen an expression library made from A431 cells and a cDNA clone encoding a 95 kilodalton protein was isolated. The function of this protein is under investigation.

Major Findings:

Regions of the EGF receptor promoter important for transcriptional activity were identified by deletion mapping and a 465 bp region (-481 to -16) was found to support transcription in transfection studies with promoter CAT fusions. We have purified a 120 kDa protein termed ETF1 that binds to GC-rich sequences in the EGF receptor promoter and stimulates its transcription. The epidermal growth factor receptor (EGFR) gene does not have a TATA box in the promoter region. ETF recognizes various GC-rich sequences including stretches of deoxycytidine or deoxyguanosine residues and GC boxes with similar affinities. ETF also binds to TATA boxes but with a lower affinity. ETF stimulated in vitro transcription from several promoters without TATA boxes, but had little or no effect on TATA box-containing promoters even though they had strong ETF-binding sites. These inactive ETF-binding sites became functional when placed upstream of the EGFR promoter whose own ETF-binding sites were removed. Furthermore, when a TATA box was introduced into the EGFR promoter, the responsiveness to ETF was abolished. These results indicate the ETF is a specific transcription factor for promoters which do not contain TATA elements.

Publications:

Jinno Y, Merlino GT, Pastan I. A novel effect of EGF on mRNA stability, Nucl Acids Res 1988;16:4957-66.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08010-16 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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	NCI, NIH
Other:	I. Pastan	Chief, Laboratory Molecular Biology	NCI, NIH
	M. M. Gottesman	Chief, Molecular Cell Genetics	NCI, NIH
	F. Thiebaut	Visiting Fellow	NCI, NIH
	K. Chang	Visiting Fellow	NCI, NIH
	U. Germann	Guest Researcher	NCI, NIH

## COOPERATING UNITS (if any)

Molecular Biology Section	LMB, NCI, NIH
Molecular Cell Genetics Section	LMB, NCI, NIH

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Ultrastructural Cytochemistry Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

4.0

## 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 unexpanded type. Do not exceed the space provided.)

Neoplastic transformation produces many changes in cell function, some of which can be studied using morphologic techniques. We have used morphologic methods to isolate monoclonal antibodies to tumor-specific epitopes for use in therapy of human cancers of ovary, colon, breast and stomach. The evaluation of such new antibodies and their tumor and tissue specificity has led to the isolation of some that may be useful in cancer therapy and diagnosis. One of these, OVB3, is currently in clinical trials as part of an immunotoxin for the treatment of ovarian cancer. Two new antibodies, OVM3 and OVM4, are being evaluated. We are continuing to study new strategies for screening and immunization for monoclonal antibody production to tumor-specific antigens. We have also evaluated antibodies directed at the human multidrug transporter, including the cross-reactivity of antibody C219 to myosin isoforms and the reactivity of a new antibody, MC57. In addition, we have examined the ability of the multidrug transporter to pump protons out of cells by measuring the alkalization of the cytosol using fluorescence photometry and microinjection of pH-sensitive dyes.

Major Findings:

Neoplastic transformation produces many changes in cell function, some of which are likely to be expressed as alterations in surface antigens. We have continued development of methods in an attempt to isolate tumor-specific monoclonal antibodies that could be used as part of immunotoxins for cancer therapy. We have used morphologic screening with both immunofluorescence and immunoperoxidase to evaluate the specificity of isolated monoclonal antibodies, methods that were used to isolate an earlier ovarian-specific antibody, OVB3, that is now in clinical testing. We have expanded our search for tumor-specific antigens to include breast, gastric, and colon cancers, as well as to ovarian cancers. In addition to using these other tumors in initial screening, we are beginning immunizations of mice with cell lines derived from these other tumors, including HF-29 cells from colon carcinoma and MCF-7 cells from breast carcinoma. We have also begun to use new strategies for immunization including: 1) tolerization of mice with normal human tissue extracts along with cyclophosphamide treatment, 2) periodate-mediated conjugation of anti-mouse IgG to the surface of immunizing cells, and 3) the evaluation of panning of lymphocytes for tumor cell-reactivity prior to fusion. We have also begun to use different timing protocols for immunization and to combine both regional lymph node and spleen cells for fusions. An additional method is the use of various acid wash procedures to eliminate low affinity binding of immunoglobulins both from conditioned media and supernatants, especially when using HT-29 cells that possess IgA receptors. Using these methods, we have isolated two new antibodies, OVM3 and OVM4, that are currently being evaluated for their tissue and tumor reactivity.

Another effort is the evaluation of tumor-specific antibodies isolated by other laboratories. We have evaluated several that have shown previously undetected reactivity with important normal tissues. Several others are still under evaluation. So far, we have not found antibodies with better specificity for tumor versus normal tissues than OVB3.

In other studies, we have evaluated the reactivity of several antibodies reactive to P170, the multidrug transporter (P-glycoprotein). One of these, C219, had previously shown reactivity to slow-twitch and cardiac muscle, and the 200 kD protein band reactive with this antibody suggested that the reactive protein was an isoform of myosin. Subsequently, we have purified myosin from cardiac muscle based on its salt fractionation and lack of membrane association, and derived a relatively pure preparation of myosin by these standard methods. This purified material was shown to react with C219 in the same way as the 200 kD band in crude extracts of cardiac muscle. Further preliminary studies using protease cleavage of myosin have suggested a reactive site in the S2 region of this myosin isoform.

As part of a collaborative project, we have also evaluated a new anti-P170 antibody isolated in Italy, termed MC57, which showed the same morphologic distribution of P170 seen in previous studies using monoclonal antibody MRK16, both in tissues and cultured cells. The relative affinity of this antibody for P170 appeared lower than MRK16. We have also, using immunocytochemistry,



evaluated the location of P170 in various systems that express the human multidrug transporter. One of these systems, which involves the expression of human P170 in a baculovirus-mediated transfer of the human MDR1 gene into insect cells showed a high level of expression of P170 in Golgi and plasma membranes, consistent with its location in human cells that normally express the protein.

In other studies, we noticed the rapid acidification of the culture medium by multidrug-resistant cells and investigated whether P170 in these cells might function as a proton pump, alkalinizing the cytosol. We have evaluated the pH of the cytosol in multidrug resistant cells using direct mechanical microinjection of a dextran-coupled dye, SNARF, that changes its spectral fluorescence properties in response to pH in the range of 7.0-8.0. Using fluorescence photometry of single living cells, we have shown that the pH in MDR cells is significantly elevated, and that this elevation of cytosol pH is enhanced by the inclusion of colchicine in the medium. On the other hand, incubation in competitive blockers of P170 pump activity, such as verapamil and vinblastine, inhibit this elevation in cytosol pH. These results suggest that an endogenous substrate for the multidrug transporter exists in cells, that active pumping of colchicine by P170 indirectly pumps protons out of the cell, and that high affinity competitors of drug binding to P170 are highly effective at inhibiting efflux of other drugs while being pumped themselves in relatively small amounts. These results add new insights into the physiological role that the multidrug transporter may play in physiology.

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Strange K, Willingham MC, Handler JS, Harris HW. Apical membrane endocytosis via coated pits is stimulated by removal of antidiuretic hormone from isolated, perfused rabbit cortical collecting tubule, *J Membrane Biol* 1988;103:17-28.

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Patent:

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08011-15 LMS

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Structures and Roles of Transformation-Sensitive Cell Surface Glycoproteins

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

PI: K. M. Yamada Chief, Membrane Biochemistry Section LMB, NCI

Other:	S. Aota	Visiting Fellow	LMB, NCI
	S. Shinagawa	Guest Researcher	LMB, NCI
	T. Nagai	Guest Researcher	LMB, NCI
	S. K. Akiyama	Guest Researcher	LMB, NCI
	K. Olden	Guest Researcher	LMB, NCI
	Y. Yasuda	Guest Researcher	LMB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Membrane Biochemistry Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.1

## PROFESSIONAL:

3.2

## OTHER:

0.9

## CHECK APPROPRIATE BOX(ES)

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- (a1) Minors
- (a2) Interviews

B

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

Fibronectin and other extracellular molecules play crucial roles in cell adhesion, migration, invasion, and metastasis. Four key sequences in fibronectin involved in its recognition by cells are being characterized. One site containing the Gly-Arg-Gly-Asp-Ser sequence was found to be required for epithelial as well as fibroblastic cell adhesion to fibronectin. A synthetic peptide containing this sequence inhibits experimental metastasis, and its overly rapid rate of renal clearance was decreased by coupling it to a carrier. A second, synergistic cell-interaction sequence was defined by recombinant DNA mutagenesis approaches. This second site was found to be required for efficient cell adhesion, transmembrane effects on the cytoskeleton, and cell migration; its crucial minimal sequence is being defined. A third site, present in only some fibronectin molecules due to alternative splicing, was recognized in cell-type specific fashion by neural crest cells and derivatives. Crest cells used it in combination with the first two sites for cell migration. Vitronectin, another major cell adhesion protein, was characterized biochemically and was found to be regulated by an extracellular molecule termed PG-M. This novel proteoglycan can modulate adhesion via its chondroitin sulfate side chains. The mechanisms of human tumor cell migration and invasion on various extracellular substrates are being analyzed in detail using a variety of potential synthetic peptide inhibitors and by assays of migration such as time-lapse video microscopy.



### Major Findings:

Fibronectin is a major cell surface and extracellular matrix glycoprotein involved in cell adhesion and migration, and is viewed as the prototype cell surface interaction protein. Knowledge of the peptide sequences essential for cell interactions with fibronectin and other extracellular proteins should aid in the development of specific inhibitors of migration, invasion, and metastasis.

The essential polypeptide sequences required for normal and tumor cell interactions with fibronectin are being identified by site-directed mutagenesis and synthetic peptide analyses. Four crucial polypeptide sites were identified: two are used by a variety of cell types and are synergistic, and two are cell-type specific and alternatively spliced. One site contains the previously described Gly-Arg-Gly-Asp-Ser sequence, which was demonstrated to be required for epithelial cell adhesion besides its known roles in fibroblast adhesion. We had previously shown that this peptide inhibits experimental metastasis and prolongs survival in a mouse melanoma model; coupling Gly-Arg-Gly-Asp-Ser-Pro-(Ala) derivatives to IgG or to a synthetic hydrophilic polymer was found to retard substantially the presently unacceptably rapid loss of such peptides from the circulation due to renal clearance.

A second, synergistic sequence in fibronectin was shown to be a novel region required for fibronectin receptor binding, efficient cell adhesion, and transmembrane effects of fibronectin on the cytoskeleton. The crucial amino acids involved have been narrowed down by oligonucleotide-directed deletion mutagenesis to 38 residues in an evolutionarily conserved part of the protein. The minimal sequences necessary for function will be determined by further mutagenesis, then tested as synthetic peptide inhibitors of cell adhesion and migration.

Both of these synergistic sites, plus a third, cell-type specific site we previously identified (termed CS-1), were found to be required for locomotion by embryonic neural crest cells. Experiments using recombinant wild-type and mutated polypeptides and synthetic peptides demonstrated that this highly migratory cell type uses different combinations of these sites in differing fashions for cell adhesion, spreading, and translocation; cell migration was particularly demanding in requiring the presence of all three of these sites.

Another major cell adhesion protein termed vitronectin was characterized biochemically and was found to exist in two structurally and functionally distinct forms in human plasma. One form is monomeric, while the other contains a proteolytically clipped product in 6.5 S aggregates that can interact with heparin and which are increased during blood coagulation. The existence of these two forms may permit distinct functions for a single gene product.

Besides depending on specific sequences and rates of synthesis, the functions of cell adhesion molecules may also be regulated by extrinsic molecules. A newly described cell surface proteoglycan termed PG-M was found to regulate the adhesion of a wide variety of cells to fibronectin, collagen, vitronectin, and laminin. Inhibitory activity was localized to the chondroitin sulfate side chains of this novel proteoglycan.

A comparative analysis is being completed of potential synthetic peptide inhibitors of human tumor cell migration using seven distinct peptide sequences previously reported to be inhibitors of cell interactions with fibronectin, collagen, and laminin, using cell outgrowth assays and time-lapse video microscopy on various extracellular proteins. Inhibitory activity has been found so far only in those derivatives containing the fibronectin Arg-Gly-Asp sequence. Methods are now being developed for the direct visualization and high-resolution video filming of specialized cell surface structures (invadopodia) actively involved in tumor cell migration and invasion of extracellular matrices.

Publications:

Humphries MJ, Yasuda Y, Olden K, Yamada KM. The cell interaction sites of fibronectin in tumor metastasis, CIBA Symp 1988;141:73-93.

Obara M, Kang MS, Yamada KM. Site-directed mutagenesis of the cell-binding domain of human fibronectin: Separable, synergistic sites mediate adhesive function, Cell 1988;53:649-657.

Dufour S, Duband J-L, Humphries MJ, Obara M, Yamada KM, Thiery JP. Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules, EMBO J 1988;7:2661-71.

Yamada KM. Fibronectin domains and receptors. In: Mosher DF, ed. Fibronectin. New York: Academic Press, 1988, 47-121.

Nishida T, Nakagawa S, Watanabe K, Yamada KM, Otori T, Berman MB. A peptide from fibronectin cell-binding domain inhibits attachment of epithelial cells, Invest Ophthalmol 1988;29:1820-25.

Izumi M, Yamada KM, Hayashi M. Vitronectin exists in two structurally and functionally distinct forms in human plasma, Biochim Biophys Acta 1989;990:101-3.

Yamagata M, Suzuki S, Akiyama S, Yamada KM, Kimata K. Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates, J Biol Chem 1989; in press.

Humphries MJ, Olden K, Yamada KM. Fibronectin and cancer: Implications of cell adhesion to fibronectin for tumor metastasis. In: Carson S, ed. Fibronectin in Health and Disease. Boca Raton, FL: CRC Press, 1989, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08705-13 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Genetic and Biochemical Analysis of Cell Behavior

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

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

Other: S. Goldenberg Research Chemist LMB, NCI  
 R. Fleischmann Staff Fellow LMB, NCI  
 D. Ray Research Biologist 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:

1.5

## PROFESSIONAL:

1.5

## OTHER:

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 are utilizing the Chinese hamster ovary (CHO) fibroblast and human cervical adenocarcinoma cell (KB) to study the genetics and biochemistry of some aspects of the behavior of cultured cells. Our recent work has emphasized the manner in which cyclic AMP regulates cell growth and gene expression in CHO cells and the function of EGF receptors in KB cells. The mechanism of cAMP action on CHO cells has been studied by isolating CHO mutants resistant to growth inhibition by cAMP. These mutants have defective cAMP dependent protein kinases with either altered regulatory (RI) or catalytic (C) subunits. DNA from cells carrying dominant cAMP-resistant defects can be used to transfer the cAMP-resistance phenotype to sensitive cells. Cosmid clones carrying the CHO RI subunit from cAMP resistant cells have been isolated. The exon-intron structure of most of the CHO RI subunit gene has been determined. A full-length cDNA for CHO RI has been isolated and sequenced. The yeast phosphodiesterase has also been expressed in CHO cells. Expression of the PDE gene results in insensitivity to cAMP. KB cells selected for resistance to a conjugate of Pseudomonas toxin with EGF have reduced levels of EGF receptors due to a decrease in EGF receptor mRNA. Many of these mutants are dominant in gene transfer experiments, and some show reduced growth in response to EGF.

Major Findings:

1. We have isolated cosmid clones from CHO libraries for the RI gene. Sequence analysis of one RI genomic clone, pcosRI, confirms that it contains most of the coding sequence of CHO RI. The RI gene has at least seven coding exons, all of which have been completely sequenced. Two exons are within the cAMP binding domain at the 3' end of the gene.
2. Some cAMP dependent protein kinase mutants are supersensitive to natural product cytotoxic drugs such as colchicine, vinblastine, adriamycin and puromycin. Revertant cells which have recovered normal levels of drug-resistance have lost their protein kinase mutant phenotype. This result suggests that the expression of resistance to multiple drugs can be modulated by cAMP dependent protein kinase, perhaps through a mechanism involving the multidrug transporter (mdr2 gene product). In confirmation of this hypothesis, CHO protein kinase mutants were found to have reduced levels of mdr RNA and reduced amounts of the transporter, indicating that in CHO cells, AMP dependent protein kinase modulates mdr mRNA levels.
3. Several classes of pleiotropic mutants resistant to a conjugate of EGF with Pseudomonas toxin have been isolated. These mutants have reduced amounts of EGF receptor mRNA. 4/5 mutants are dominant in somatic cell hybrids and the defect in 2 of the dominant mutants has been transferred by DNA-mediated gene transfer. The growth of KB cells in serum-free medium is stimulated by EGF, but in 3/5 of the mutants with low EGF receptor levels, little or no stimulation of growth by EGF is seen. 4/5 mutants also had a reduced growth response to insulin. These results indicate that reduction of EGF receptor RNA in most of these mutants is part of a pleiotropic defect affecting response to other growth factors as well as EGF.

Publications:

- Amano F, Gottesman MM, Pastan I. Epidermal growth factor-dependent growth of human KB cells in a defined medium and altered growth factor requirements of KB mutants resistant to EGF-Pseudomonas conjugates, J Cell Physiol 1988;135:502-8.
- Amano F, Pastan I, Gottesman MM. Genetic characterization of human KB cell lines resistant to epidermal growth factor: Pseudomonas exotoxin conjugates, J Cell Physiol 1988;135:527-32.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08710-13 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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:

1.0

## PROFESSIONAL:

1.0

## 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.)

Two major questions of DNA replication are being studied: First, what are the molecular mechanisms involved in the initiation of replication? Second, how is DNA replication regulated? To address these questions, I have been using an in vitro replication system that replicates plasmid DNA carrying the bacteriophage P1 origin of replication. I have determined many host functions that participate in the replication reaction, including dnaA, dnaB, dnaC and dnaG proteins, RNA polymerase, DNA gyrase and DNA polymerase III. I have found that three of the E. coli heat shock proteins, dnaJ, dnaK, and grpE proteins are also required. By studying protein-protein and protein-DNA interactions among these proteins, I am learning how they assemble on the initiation site and how they function in initiation. Since P1 normally exists stably as a unit copy plasmid, this system is being used to study the molecular mechanisms involved in the regulation of a stringently controlled replicon. I have found that with reaction conditions that allow the replication of DNA carrying the origin region, plasmid DNA carrying in addition the control region is not replicated. In vitro, the P1 initiator protein, RepA, binds to the origin and control region simultaneously, causing the intervening DNA to loop as determined by electron microscopy. Our interpretation is that this looping inhibits initiation. Replication in vitro is restored when seven or more of the nine RepA binding sites are deleted from the control region or when wild-type RepA protein is substituted with RepA protein isolated from a repA mutant that has an increased copy number in vivo. I am currently trying to understand what factors or conditions regulate looping such that replication occurs once per cell cycle.

Major Findings:

The mini-P1 replicon consists of an origin of replication, a replicon specific initiator protein, RepA, and a control locus. The origin contains five direct repeats of a 19 bp sequence to which RepA protein binds. About 100 bp to the left there are two direct repeats of the 9 bp dnaA protein binding site. Plasmids containing this origin are maintained at high copy number. The replication control region, located 1 kb to the right of the origin, is required to maintain mini-P1 plasmids at one copy per E. coli chromosome. This region contains nine repeats of the RepA binding site. The obvious mechanism of regulation by titration of RepA by the control locus is ruled out by the finding that RepA is autoregulated.

We have cloned the origin with and without the control region in M13 and tested the RF DNA for RepA-dependent replication in vitro. DNA containing the origin alone is replicated in vitro in a reaction requiring two origin specific DNA binding proteins, dnaA and RepA. RNA polymerase, DNA gyrase, dnaB, dnaC and dnaG proteins are also required. I have recently found that three E. coli heat shock proteins, dnaJ, dnaK and grpE are essential. Furthermore, dnaJ and RepA can be isolated from cells as a protein complex. The dnaJ-RepA complex can be reconstituted in vitro by mixing the two purified proteins and subjecting the mixture to gel filtration. The complex contains equimolar amounts of the two proteins and the native molecular weight of the complex suggests that it contains a dimer of each protein. The role of this complex in replication is not known but it is likely that this is a mechanism by which the plasmid directs the host replication machinery to the plasmid origin. I am now isolating protein-DNA complexes to understand how the nucleoprotein complex is assembled at the origin and how the proteins function in initiation.

Using reaction conditions that allow the replication of DNA carrying the origin region, plasmid DNA carrying in addition the control region is not replicated in vitro even when RepA is added in a ten-fold excess relative to binding sites. In collaboration with Dhruba Chatteraj (NCI, NIH), we have seen by electron microscopy that RepA binds to the origin and control region simultaneously, causing the intervening DNA to loop. Our interpretation is that this looping inhibits initiation. In collaboration with Keith McKenney (CARB, NIST), we have constructed deletions of the control locus. We have sequenced the deletions and tested the DNAs for their ability to support RepA dependent replication in vitro. DNA synthesis in vitro is restored when the distal seven or more RepA binding sites are deleted from the control region. We are currently testing these DNAs for RepA dependent DNA looping in vitro to make a correlation between initiation and inhibition of looping. Dhruba Chatteraj has isolated mutants in the repA gene that are maintained at high copy number in vivo. I have purified one of the mutant RepA proteins and found that replication of P1 plasmid DNA containing the origin and control locus is restored when the mutant RepA protein is substituted for the wild-type protein. We are now determining whether or not the mutant RepA protein is able to cause DNA looping. I am also trying to determine conditions and identify factors that regulate looping such that replication occurs once per cell cycle.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08714-12 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Bacterial Functions Involved in Cell Growth Control

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

PI:	S. Gottesman	Chief, Biochemical Genetics Section	LMB, NCI
Other:	S. Dasgupta	Visiting Fellow	LMB, NCI
	D. Gutnick	Visiting Scientist	LMB, NCI
	M. Maurizi	Senior Staff Fellow	LMB, NCI
	J. Trempy	Guest Researcher	LMB, NCI

## COOPERATING UNITS (if any)

Laboratory of Genetics, Division of Cancer Biology and Diagnosis		
	S. Rudikoff	LG, NCI
	J. Pumphrey	LG, NCI

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Biochemical Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.9

## PROFESSIONAL:

8.9

## 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 unabbreviated 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 the proteases which carry out ATP-dependent proteolysis in *E. coli*. The Lon protease has previously been shown to degrade RcsA, a positive regulator of capsule synthesis, and SulA, a cell division inhibitor. We have continued the analysis of capsule synthesis. RcsC, a membrane protein, probably functions to sense environmental signals and transmits this information to the positive regulatory protein RcsB. RcsA may act directly on the regulated genes, or indirectly by affecting RcsB-RcsC interactions. The Clp protease, a second major ATP-dependent protease, has an ATP-binding subunit (ClpA) which shares homology with a family of proteins in both eukaryotic and prokaryotic cells. The protease subunit of the Clp protease (ClpP) contains a serine active site, and has only limited activity in the absence of ClpA. Clp is induced anaerobically; one target of Clp degradation is excess ClpA subunit. A third energy-dependent protease, the Alp protease, is capable of substituting for Lon under some conditions.

Other Personnel:

W. Clark	Research Chemist	LMB, NCI
N. Trun	Guest Researcher	LMB, NCI
V. Stout	Guest Researcher	LMB, NCI
S. H. Kim	Visiting Fellow	LMB, NCI

Major Findings:

1. We have continued our analysis of the regulation of capsular polysaccharide synthesis. The genes for RcsB, a positive regulator of capsule, and RcsC, a membrane protein which exerts a negative regulatory effect on capsule, have both been sequenced. The predicted protein sequence confirms the predictions made from genetic studies that these proteins are member of the ubiquitous regulatory effector-sensor types found in both gram positive and gram negative bacteria. The sequence similarities with other sensors and effectors suggests a possible mode of interaction for RcsC and RcsB through phosphorylation. The sequence of rscC confirms its topology in the inner membrane as predicted from fusions to phoA. The protein crosses the inner membrane twice, and has a large cytoplasmic domain which presumably interacts with RcsB. The sequencing also suggested that rscB synthesis might be under the control of an unusual sigma factor, the product of the rpoN gene. This prediction has been confirmed, and suggests the possibility of other proteins participating in rscB regulation. The relationship of this regulation to the direct regulation of capsule synthesis is still unclear.

2. RcsA, the unstable positive regulator of capsule synthesis, has been overproduced under control of a T7 promoter. This overproduction will allow the isolation of antibody to RcsA and the development of sensitive in vivo measurements of RcsA stability. In addition, we hope to be able to reconstruct the regulatory circuit for capsule synthesis in vitro; purification of RcsA will undoubtedly be a necessary part of such a project. RcsB can also be overproduced in a similar manner. rscA\*, a mutant RcsA which increases capsule synthesis even in cells containing the Lon protease (which normally degrades RcsA rapidly and keeps capsule synthesis low) has been shown to contain a mutation in the C-terminal half of the protein. Therefore, this mutation does not affect synthesis, but may affect either activity or stability of RcsA.

3. We have continued our studies on the Clp energy dependent protease of E. coli. Sequencing of the clpA subunit has demonstrated the existence of two ATP binding domains within this 84,000 dalton protein. ClpA is highly homologous to another protein in E. coli, as well as proteins from a variety of both prokaryotic and eukaryotic organisms. All of these proteins share the two ATP-binding domain structure, as well as extensive homology throughout most of the protein. ClpA is the only protein in this group with a known function: ATPase activity and participation as the regulatory subunit of an energy-dependent protease. The existence of this family of conserved proteins suggests the existence of Clp-like proteases in many organisms. The gene for the ClpP subunit of the protease has also been cloned, mapped and inactivated. The gene appears to be dispensable for E. coli. It maps about 2 kb upstream of



the gene for the Lon ATP-dependent protease, but ClpP has relatively little similarity in structure to Lon, and there as yet is no evidence of co-regulation of these proteases. The active site serine of ClpP has been identified by labeling with radioactive DFP; site-directed mutagenesis of this site is also being carried out.

4. We have identified as substrates for the Clp protease the ClpA subunit itself and ClpA-lacZ fusion proteins. In addition, lacZ fused to genes in the ribose operon also creates fusion proteins which are subject to Clp-dependent degradation. Since the degradation of the LacZ moiety in these fusion proteins provides a color test for degradation in vivo, we are in a position to analyze the targets of Clp recognition by selecting and sequencing stable mutants. Use of the clpA-lacA fusion has also indicated that clpA synthesis is anaerobically controlled, and increases dramatically as cells enter late logarithmic growth. Deletion analysis of the promoter and isolation of trans-acting regulators should identify the molecular basis for this regulation. These observations suggest that the natural substrates for Clp degradation may be found after a shift from aerobic to anaerobic growth.

5. N protein of bacteriophage  $\lambda$  can be degraded by Lon protease in a purified in vitro system. We have begun directed mutagenesis of the cleavage sites in N to analyze the basis of Lon recognition of its substrates.

6. The Alp proteolytic system, identified as a function which suppresses lon mutants when present on multicopy plasmids, has been shown to increase energy-dependent degradation of the Lon substrate Sula. Since this activity is independent of either Lon or Clp, it seems likely that Alp increases the activity of yet another energy-dependent protease in E. coli. Sequencing of alp reveals a short open reading frame. It seems likely that the alp function present on multi-copy plasmids is only the limiting component or regulator of a larger protease. We can use the genetic screens which led to alp identification to isolate mutations in these other components.

7. We have begun the isolation of E. coli mutations which interfere with proper chromosome partitioning. By selecting cells which have increased chromosome number, candidate mutations have been isolated and are being mapped and further characterized.

#### Publications:

Brill JA, Quinlan-Walsh C, Gottesman S. Fine-structure mapping and identification of two regulators of capsule synthesis in Escherichia coli K-12, J Bacteriol 1988;170:2599-2611.

Katayama Y, Gottesman S, Pumphrey J, Rudikoff S, Clark WP, Maurizi MR. The two-component ATP-dependent Clp protease of Escherichia coli: purification, cloning, and mutational analysis of the ATP-binding component, J Biol Chem 1988;263:15226-15236.

Maurizi MR, Katayama Y, Gottesman S. Selective ATP-dependent degradation of proteins in Escherichia coli. In: Schlesinger MJ, ed. The Ubiquitin System: Current Communications in Molecular Biology. Cold Spring Harbor NY: Cold Spring Harbor Laboratory, 1989;147-154.

Trempey JE, Gottesman S. Alp: A suppressor of Lon protease mutant in Escherichia coli. J Bacteriol 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08715-11 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Synthesis and Function of a Transformation-Dependent Secreted, Lysosomal Protease

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

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

Other:	S. Goldenberg	Research Biologist	LMB, NCI
	D. Ray	Research Biologist	LMB, NCI
	B. Troen	Medical Staff Fellow	LMB, NCI
	S. Kane	Guest Researcher	LMB, NCI
	S. Smith	Guest Researcher	LMB, NCI
	A. Salminen	Visiting Fellow	LMB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Molecular Cell Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

3.0

## OTHER:

0.0

## CHECK APPROPRIATE BOXES)

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

B

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

Cultured mouse fibroblasts which are malignantly transformed or treated with TPA or growth factors such as PDGF synthesize and secrete the 39,000 Mr precursor to cathepsin L in large amounts. Overproduction of cloned cathepsin L in a nontransformed cell results in secretion of this lysosomal enzyme. Cathepsin L is an active acid protease whether or not it is glycosylated. Secreted procathepsin L can bind to the mannose 6-phosphate receptor of many cells and be endocytosed and processed intracellularly. In antigen presenting cells, procathepsin L uptake reduces the efficiency of antigen presentation, thereby interfering with immune response. Transformation, TPA and PDGF stimulate transcription. We have cloned a functional procathepsin L gene from the mouse and have identified and sequenced the 5' flanking region which acts as a promoter in CAT constructions. Regulated transcription requires sequences in the first two exons and introns. A human procathepsin L cDNA has also been cloned and sequenced. Recombinant human procathepsin L produced in bacteria is enzymatically active. A deletion analysis of procathepsin L produced in bacteria indicates that both the "pro" and the carboxy-terminal part of the protein are needed for full activity. Polyclonal and monoclonal antibodies to human cathepsin L have been prepared using the recombinant antigen.

Major Findings:

1. Procathepsin L added to antigen-presenting cells which process and present pigeon cytochrome C reduces the efficiency of antigen presentation. The mechanism of this effect involves endocytosis of procathepsin L via mannose 6-phosphate receptors. Within the cell, cathepsin L protease activity is presumed to destroy the antigen. This result suggests that one function of MEP may be to alter the immune response of tumor bearing hosts.
2. Complete coding sequences for mouse and human MEP have been obtained. The deduced amino acid sequences have been used to determine the cleavage sites in preprocathepsin L to yield the secreted procathepsin L and the processed lysosomal 29 kDa and 20 kDa forms.
3. The mouse MEP (procathepsin L) gene has been cloned and the promoter region has been isolated. A 300 bp fragment upstream from the transcription initiation site is an adequate promoter in MEP-promoter-CAT constructions. Regulated transcription requires sequences in the first two exons and introns.
4. Cloned mouse and human procathepsin L can be transferred to mouse cells and expressed at high levels using the pHaMDR1 amplification system. High levels of procathepsin L do not produce a transformed phenotype in NIH 3T3 cells, but they do result in secretion of procathepsin L, indicating that overproduction of this lysosomal protein is sufficient to result in its secretion.
5. Recombinant human procathepsin L has been produced in large quantities in E. coli. Yields of approximately 0.5 g/10L culture have been obtained. The recombinant enzyme is active after solubilization in urea and reduction/oxidation in glutathione above pH 10. A deletion analysis of the recombinant human cathepsin L shows that both amino and carboxy-terminal sequences are needed for activity. Glycosylation is not essential for activity of cathepsin L.
6. Using recombinant procathepsin L produced in E. coli, we have prepared both rabbit polyclonal antisera and mouse monoclonal antibodies to human procathepsin L. These should be useful for determination of cathepsin L levels in clinical samples.

Publications:

McCoy K, Gal S, Schwartz R, Gottesman MM. An acid protease secreted by transformed cells interferes with antigen processing, *J Cell Biol* 1988; 106:1879-84.

Kane SE, Troen BR, Gal S, Ueda I, Pastan I, Gottesman MM. Use of a cloned multidrug-resistance gene for co-amplification and overproduction of MEP, a transformation-regulated secreted acid protease, *Mol Cell Biol* 1988;8:3316-21.

Smith SM, Kane SE, Gal S, Mason RW, Gottesman MM. Glycosylation of procathepsin L does not account for species molecular weight differences and is not required for proteolytic activity, *Biochem J* 1989; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08717-11 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Functions, Structure, and Regulation of Receptors for Cell Adhesion Proteins

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

PI: K. M. Yamada Chief, Membrane Biochemistry Section LMB, NCI

Other:	S. E. La Flamme	Biotechnology Fellow	LMB, NCI
	K. Takenaga	Guest Researcher	LMB, NCI
	S. K. Akiyama	Guest Researcher	LMB, NCI
	S. S. Yamada	Guest Researcher	LMB, NCI
	H. Larjava	Visiting Fellow	LMB, NCI
	W.-T. Chen	Guest Researcher	LMB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Membrane Biochemistry Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.1

## PROFESSIONAL:

3.2

## OTHER:

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.)

Receptors for key extracellular proteins such as fibronectin, collagen, and laminin are thought to mediate adherence, migration, and invasion by tumor cells, as well as analogous processes by certain normal cells. Membrane glycoproteins that bind collagen or fibronectin are being characterized biochemically and functionally. One collagen-binding protein is a transformation-sensitive heat-shock protein, which has been characterized in terms of biosynthetic steps and partial protein sequence. A second binding protein termed VLA-2 may be central to normal and tumor cell penetration of collagenous matrices. Fibronectin receptors were shown to be altered in quantity and/or distribution *in vivo* and *in vitro*. For example, levels are increased in Rous sarcoma-virus induced tumors, and its membrane localization is altered in a variety of tumor cells. A similar alteration of distribution to a diffuse pattern is found transiently in certain rapidly migrating embryonic cells. Lateral mobility of fibronectin receptors in the membrane was high in migrating cells, but lost as cells ceased migration and established associations with fibronectin and cytoskeletal elements. The structure of human fibronectin receptors was determined by a variety of electron microscopic approaches; it has an unusual shape with two long stalk-like legs. Subunit-specific monoclonal antibodies elucidated fibronectin receptor function in a variety of processes. The beta subunit, shared by other receptors, appears required for normal cell attachment to fibronectin, laminin, and collagen. The alpha subunit provides specificity. This fibronectin receptor was required for organization of an extracellular fibronectin matrix, specialized adhesive contacts, and thick intracellular microfilament bundles. It also restrains migration of normal fibroblasts as part of its function in maintaining the stationary cell phenotype. It thus appears to play a central role in regulating normal cell adhesion and migration. Direct comparisons of its roles in migration and invasion by malignant cells are in progress.

Major Findings:

Receptors for key extracellular proteins such as fibronectin, collagen, and laminin are thought to mediate adhesion, migration, and invasion by tumor cells; they probably utilize mechanisms used transiently at certain steps in embryonic development or wound healing by certain normal cells. Characterization of these molecules should help elucidate mechanisms of invasion and metastasis, as well as providing immunological and other inhibitors.

Two collagen-binding proteins are being characterized and compared. The first is a 47,000 dalton glycoprotein we previously found to be a transformation-sensitive heat-shock protein. Its biosynthetic precursors and N-terminal sequence were characterized; further amino acid sequencing will be needed for its cloning. A second collagen-binding protein also known as VLA-2 is being characterized, and appears to be a key receptor for cell migration and invasion. The migration of both normal and malignant human cells in three-dimensional collagen gels mimicking in vivo extracellular matrices appears to require this receptor according to ongoing monoclonal antibody inhibition analyses.

Although the fibronectin receptor is hypothesized to be involved in cell migration and invasion, little was known experimentally about its functions. Three approaches were taken: receptors were localized in normal and tumor tissue, then in the plasma membrane of individual cells; the structure of the fibronectin receptor was determined by electron microscopy; and its function was analyzed by monoclonal antibodies and biochemical approaches.

The level of expression of fibronectin receptors was found to be elevated in a classical experimental tumor system. Rous sarcoma virus-induced transformation of fibrocytes to tumors resulted in a 4.7-fold elevation of fibronectin receptor family members (integrins) in chickens. The localization of the receptor was also disrupted from the normally clustered state to a diffuse distribution. Such alterations in a motility-associated receptor may help account for the invasiveness of certain tumor cells. Interestingly, in studies nearing completion of squamous cell carcinomas, integrin receptors of the fibronectin receptor family are also disrupted in localization, but overall levels are decreased. It is likely, therefore, that integrin levels will be found to be altered in malignancy-specific fashion, and that their distributions may be of particular importance. Preliminary in vitro functional studies using monoclonal antibodies indicate that integrins are important for the migration of human tumor cells; fibronectin receptors themselves are needed for migration of some, but not other, tumor cells. Contrary to a recent report of decreased fibronectin receptors in transformed rat fibroblasts in vitro, human fibrosarcoma cells and virally-transformed fibroblasts do not appear to show any decreases whatsoever in the levels of such receptors, but instead show marked alterations in their processing and distribution. Malignancy-associated alterations in integrins thus appear to be complex and deserve more careful evaluation.

The significance of the diffuse versus clustered states of the receptor was further evaluated in an unusual normal cell type. Transiently in development,

very rapidly migrating embryonic cells also display a diffuse pattern fibronectin receptors, which become clustered in focal contacts and streaks as the cells mature and cease migration. The lateral mobility of these receptors in the plane of the plasma membrane was measured by the fluorescence photobleaching method. Their lateral mobility was high on rapidly migrating cells, indicating relative freedom of motion, while mobility was quite low when located in clusters on stationary cells. Their mobility apparently changes as they associate with cytoskeletal elements and fibronectin fibrils when migration ceases.

The structure and dimensions of isolated human fibronectin receptors were determined using a variety of electron microscopic methods. Rotary shadowing, negative staining, freeze-drying, and freeze-etching were combined with computer modeling of deduced amino acid sequence data to generate a model of the receptor. This receptor has an unusual structure, with a mushroom-shaped head mounted on two long, spindly legs that protrude over 200 nm from the plasma membrane. The leg contributed by the alpha subunit appears rich in  $\beta$  strands, while that from the beta subunit contains repeating EGF-like motifs analogous to those in laminin. The model predicts that both subunits of the receptor can contribute to the ligand binding site, which appears to be confirmed by monoclonal antibody analyses of function.

Monoclonal antibodies against alpha and beta subunits of the human fibronectin receptor were used to elucidate its major functions in normal human fibroblasts. The beta subunit is shared by a variety of integrin receptors, and antibodies against it inhibit cell adhesion to fibronectin, laminin, and collagen. The alpha subunit appears to be required for initial cell adhesion to fibronectin, but not for some of later adhesive steps. It is required for cell surface retention and assembly of an extracellular fibronectin matrix, as well as for the establishment of specialized adhesive contacts under cells (focal and ECM contacts) and partially for organization of thick microfilament bundles. In contrast to its apparent role in cell migration of malignant fibroblastic derivatives, this receptor in normal fibroblasts is involved instead in restraining migration, presumably through the specialized adhesive contacts. These results suggest a central role for the fibronectin receptor in regulating cell adhesion and migration. Direct comparisons of its roles in migration and invasion by malignant cells are in progress.

#### Publications:

Nagata K, Hirayoshi K, Obara M, Saga S, Yamada KM. Biosynthesis of a novel transformation-sensitive heat-shock protein that binds to collagen, *J Biol Chem* 1988;263:8344-49.

Nagata K, Saga S, Yamada KM. Characterization of a novel transformation-sensitive heat-shock protein (HSP47) that binds to collagen, *Biochem Biophys Res Commun* 1988;153:428-434.

Saga S, Chen W-T, Yamada KM. Enhanced fibronectin receptor expression in Rous sarcoma virus-induced tumors, *Cancer Res* 1988;48:5510-13.

Nermut MV, Green NM, Eason P, Yamada SS, Yamada KM. Electron microscopy and structural model of human fibronectin receptor, *EMBO J* 1988;7:4093-99.

Duband J-L, Nuckolls GH, Ishihara A, Hasegawa T, Yamada KM, Thiery JP, Jacobson K. Fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells, *J Cell Biol* 1988;107:1385-96.

Akiyama SK, Yamada SS, Chen W-T, Yamada KM. Analysis of fibronectin receptor function with monoclonal antibodies: Roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization, *J Cell Biol* 1989, in press.

Yamada KM, Yamada SS. Isolation of fibronectin receptors. In: Litwack G, ed. *Receptor Purification*. Clifton NJ: Humana Press, 1989, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08719-09 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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: Bruce H. Howard Chief, Molecular Genetics Section LMB, NCI

Other: M. Fordis	Expert	LMB, NCI
A. Giordano	Biotechnology Fellow	LMB, NCI
D. Jones	Biotechnology Fellow	LMB, NCI
T. Howard	Guest Researcher	LMB, NCI
K. Sakamoto	Visiting Fellow	LMB, NCI
S. Goldstein	Visiting Associate	LMB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Molecular Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

6.0

## PROFESSIONAL:

5.0

## OTHER:

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 primary focus of work in the Molecular Genetics Section continues to be on growth regulation in mammalian cells with investigation of growth inhibitory activity emphasized within this framework. Several results of interest have been obtained. i) Growth regulatory properties of 7SL RNA and Alu elements have been shown to reside in two short DNA sequence motifs: the B block of the RNA polymerase III promoter and an undecanucleotide, GAGGCNGAGGC, which is homologous to papovavirus origins of DNA replication. ii) Screening of an Okayama-Berg cDNA expression library has led to identification of several clones differentially expressed in serum-starved and senescent human skin fibroblasts. iii) A new procedure for construction of full length cDNA expression vector libraries has been developed.

Major Findings:

- I. New methods to improve DNA-mediated stable transformation efficiencies. This project is currently inactive; results are in press.
- II. Application of gene transfer methods to detection of genes that regulate cell growth.
  - A. Development of transfection/FACS assay: The development phase of this project has been completed.
  - B. Construction of cDNA expression vector libraries. The unavailability of full length cDNA expression vector libraries from non-proliferating normal cells has been a major impediment to the isolation of growth inhibitory genes. To address this problem, a new procedure for construction of such libraries was developed. This procedure incorporates several strategies to enrich for complete cDNA copies: i) mRNA/cDNA hybrid products of first strand synthesis are selected by magnetic anti-CAP monoclonal antibody to eliminate RNA templates degraded before or during reverse transcription; ii) a synthetic primer for reverse transcriptase is used so that, following linker addition, only cDNA products with a correct 3' end will generate appropriate cohesive ends for cloning; and iii) internal restriction sites are protected by DNA methylases prior to cleavage of linkers. The feasibility of the new cDNA cloning procedure has been confirmed using a  $\beta$ -globin mRNA model system.
  - C. Inhibition of HeLa cell replication: This project continues to be the central focus of research in the the MGS. We are investigating the role(s) of RNA polymerase III (polJIII)-transcribed genes, specifically the 7SL RNA/Alu family, in regulating mammalian cell growth. Over the past year, the majority of experiments on 7SL RNA and Alu elements have concentrated on mapping small DNA sequence motifs responsible for growth regulatory properties of these sequences.

In view of the numerous correlations between pol III transcription and growth regulation, we were interested in determining whether the B block of the pol III promoter was required for either Alu- or 7SL RNA-mediated inhibition of HeLa cell  $^3\text{H}$ -thymidine incorporation. This question was approached by constructing B block-defective mutant derivatives from 7SL RNA and Alu clones. A series of *in vitro* transcription experiments using HeLa cell extracts confirmed that these mutations reduced pol III promoter activity in the respective constructs.

When wild type and B block defective derivatives were compared in DEAE-dextran transfection assays, it was found that both B block mutations completely abolished the capacity to inhibit  $^3\text{H}$ -thymidine incorporation. From these results we concluded that a pol III promoter signal was required for 7SL RNA- or Alu-mediated suppression of  $^3\text{H}$ -thymidine incorporation.

An important possibility to consider in these experiments was that intracellular 7SL RNA levels, and hence signal recognition particle (SRP) function, could be substantially altered following transfection with 7SL RNA or Alu genes. In principle, such an alteration could affect multiple cellular functions, including growth rate, DNA and protein synthesis. Northern blot and primer extension experiments were employed to demonstrate that endogenous 7SL RNA levels in the sorted cell subpopulation were unaffected by introduction of Alu sequences. Unexpectedly, total 7SL RNA levels were also unaffected by transfection with a 7SL RNA plasmid. This latter result suggests that 7SL RNA expression may be tightly regulated in HeLa cells. Pol III-dependent Alu transcription appeared to be short-lived in HeLa cells, since by 48 hrs post-transfection Alu transcripts were undetectable by either Northern or primer extension analysis of total RNA, whereas at very short times after transfection (e.g., 6 hrs) small Alu transcripts were present by Northern analysis.

Alu sequences possess several copies of the GAGGC pentanucleotide motif that is recognized by SV40 and polyoma T antigens in their respective origins of DNA replication, and this motif has been reported to promote replication of an Alu element in T antigen-containing COS cells. The 7SL RNA gene also contains one copy of the sequence GAGGCNGAGGT, which is located between the A and B block pol III promoter signals.

In order to assess whether this "ori" motif contributes to suppression of  $^3\text{H}$ -thymidine incorporation in our assay, we constructed two ori- Alu clones. The first mutant contained a 6 bp deletion that removed the left pentamer of the sequence GAGGCTGACAC; in this clone inhibitory activity was abolished. The second clone contained five point mutations within the ori motif (GAGGCTGACAC  $\rightarrow$  CATCCTTACAC); this latter mutant also exhibited reduced inhibitory activity.

We also constructed a 7SL RNA gene ori- mutant by replacing its GAGGCTGAGGT motif with the sequence GGGGCTGGGAT. The resulting ori- construct was transcribed normally in vitro, but its capacity to suppress  $^3\text{H}$ -thymidine incorporation activity was lower than the parental clone. This result and the results obtained with the two Alu ori- constructs suggest that HeLa cells contain one or more proteins which share with SV40 T antigen the capacity to interact at Alu/7SL RNA ori motifs.

To confirm that 7SL RNA/Alu family members alter growth as well as affect  $^3\text{H}$ -thymidine incorporation, growth curves on transiently transfected cell subpopulations were performed. Such growth curve assays revealed that cells which had taken up 7SL RNA or Alu plasmids divided more slowly than control cells exposed to pUC19. Doubling times of 7SL RNA- or Alu-transfected cells were extended by 1.6-fold and >4-fold relative to the control in repeat experiments. HeLa cells transfected with a 7SL RNA gene mutated in the pol III B block promoter motif grew faster than the pUC19 control.

An unexpected aspect of growth curve experiments was that  $^3\text{H}$ -thymidine incorporation was not decreased in proportion to cell proliferation in 7SL RNA or Alu transfected cultures. The reason for this anomalously high  $^3\text{H}$ -thymidine incorporation is not clear and will be the subject of future experiments; however, evidence from studies using the FACS in conjunction with propidium iodide to measure cell cycle characteristics and cellular DNA content suggest that 7SL RNA sequences can, under certain transfection conditions, induce abnormal DNA synthesis in the host cell.

In parallel with studies on the 7SL RNA/Alu family, growth regulatory properties of other DNA sequences are in progress. Transfection studies use  $\approx 500$  bp polymerase chain reaction-synthesized fragments containing synthetic arrays of DNA-binding protein recognition sites. Sites from negative regulatory regions within promoters of proto-oncogenes and other growth-associated genes, as well as positive regulatory regions within promoters of quiescence/senescence-associated genes, may provide "handles" to identify intracellular growth inhibitory proteins.

D. Regulation of growth in cell types other than HeLa. Pooled subfractions of an Okayama-Berg cDNA expression vector library from Werner syndrome fibroblasts were tested for the capacity to inhibit  $^3\text{H}$ -thymidine incorporation in normal human fibroblasts. The pool with the highest activity was then used as a probe to detect genes that are differentially expressed in Werner syndrome cells. Preliminary sequence analysis of several cDNA clones isolated by this two step protocol has identified fibronectin, pro- $\alpha 1(\text{I})$  collagen, and nuclear lamin-related mRNAs. A full length cDNA clone for the latter mRNA will be tested for growth suppression activity.

#### Publications:

Corsico CD, Howard BH. Chloramphenicol acetyltransferase as a reporter in mammalian gene transfer. In: Pollard JW, Walker JM eds. *Methods in Molecular Biology*, Vol. 5. Clifton, NJ: Humana Press, Inc., 1989; in press.

Goldstein S, Fordis CM, Howard BH. Enhanced transfection efficiency and improved cell survival after electroporation of G2/M-synchronized cells and treatment with sodium butyrate, *Nucl Acids Res* 1989; in press.

Goldstein S, Murano S, Benes H, Moerman EJ, Jones RA, Thweatt R, Shmookler Reis RJ, Howard BH. Studies on the molecular-genetic basis of replicative senescence in Werner syndrome and normal fibroblasts, *Exp Gerontol* 1989; in press.

Holter W, Spiegel AM, Howard BH, Brann MR. Expression of GTP-binding proteins during human T cell activation, *J Immunol* 1989; in press.

Howard BH. A potential role for interspersed repetitive sequence elements in negative growth regulation. In: Wang E, Warner HR, eds. *Growth control during cell aging*. Boca Raton, FL: CRC Press, Inc. 1989; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08750-09 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Genetic Regulatory Mechanisms in Escherichia coli and Its Bacteriophage

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

PI:	S. Adhya	Chief, Developmental Genetics Section	LMB, NCI
	S. Garges	Microbiologist	LMB, NCI
Other:	J. Kim	Biotechnology Fellow	LMB, NCI
	G. Storz	Biotechnology Fellow	LMB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Developmental Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.6

## PROFESSIONAL:

2.6

## OTHER:

0.0

## CHECK APPROPRIATE BOXES)

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

B

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

Cyclic AMP binds to its receptor protein CRP, and induces a conformational change (allosteric transition) in the protein. Cyclic AMP·CRP complex, and not free CRP, binds at or near many promoters of E. coli in a sequence-specific way and either represses or activates transcription. We have isolated and characterized several classes of mutations in the crp gene, which identify the amino acids participating in the cyclic AMP-induced allosteric shift in the protein. On the basis of these results and the three-dimensional structure of the protein, we proposed that cyclic AMP brings about: (1) a hinge reorientation to release the DNA-binding F  $\alpha$ -helices; (2) proper alignment between two subunits; (3) an adjustment between the position of the two domains in each subunit. Extensive amino acid substitutions at some of these positions further supports our idea that a hinge movement involving specific interhelical polar-polar interactions and Van der Waals repulsions between side chains of defined amino acids are an essential part of the activation of CRP. We have obtained mutants that are in a allosterically frozen conformation that is uninducible by cAMP. These mutations impart a defect in DNA binding and/or in transcription activation. This suggests that cAMP-induced allosteric change is necessary not only for DNA binding but also for CRP-RNA polymerase interaction. We are also studying the proposed CRP-RNA polymerase interaction when bound to DNA by isolating mutants of CRP that are altered in this interaction and by changing the distance between the corresponding DNA binding sites. The results suggest a specific DNA distance between the two bound proteins for productive interaction. It is likely that there is a complicated arrangement between the two sites, in terms of DNA sequence and distance. We have found that CRP exerts an effect on the translation of the galE gene.

Major Findings:

CRP: We are studying the cAMP receptor protein (CRP), a protein that binds to DNA and can activate or transcription of many operons of Escherichia coli. Study of such a protein provides a model system for investigations of transcriptional activators and repressors, and provides information on protein-protein interactions, DNA-protein interactions, and protein conformation.

We are studying three aspects of CRP:

A. The cAMP-induced allosteric change.

CRP is inactive for site-specific DNA binding unless cAMP is bound. Cyclic AMP binding to CRP causes the protein to undergo a conformational change. We have previously isolated several classes of CRP mutants that are allowing us to define how cAMP induces the allosteric change. We have found that the hinge region connecting the cAMP-binding large domain to the DNA-binding small domain is an essential participant in the cAMP-induced change.

1. The amino acid at the apex of this hinge in the wild-type protein is aspartic acid. Using oligonucleotide-directed site-specific mutagenesis, we have changed this amino acid to many other amino acids. We have studied the in vivo characteristics of these mutants; in addition, the mutant proteins were purified and subjected to various biochemical analyses. These analyses included DNA binding affinity, cAMP binding affinity, and sensitivity of proteolysis. Our results show that all mutants bind cAMP normally, but four of the mutants have 100-fold less affinity for DNA compared to wild type. Two of these four mutants are still active in vivo when overproduced and by proteolysis show the normal CRP pattern with and without cAMP. The other two mutants are absolutely inactive in vivo, even when overproduced, and show a conformation, even in the presence of cAMP, like that of CRP without cAMP. These latter two mutants appear to be frozen in an intermediate conformation that is defective in DNA binding and transcription activation. When overexpressed they bind to the CRP binding site on DNA but still cannot activate transcription. This suggests that the cAMP-induced allosteric change is necessary not only to achieve higher affinity specific DNA binding, but also to allow CRP to activate transcription.

2. We have continued studying a particular cAMP-independent mutant that has changes at amino acids 142 and 144, around the hinge region. We have performed detailed biochemical analyses of the purified protein. In studying cAMP affinity for the mutant, it was found that cAMP binding was positively cooperative, unlike cAMP binding to wild type CRP which shows slight negative cooperativity. The wild type CRP shows cooperative binding to cAMP only in the presence of DNA. This suggests that this mutant protein might have a conformation that normally CRP achieves only upon DNA binding.

#### B. How CRP activates transcription.

CRP most likely activates transcription through interaction with RNA polymerase. Placement of the CRP binding site relative to the promoter is apparently very important. We looked for DNA insertion between the CRP binding site and the promoter that would still allow activation by CRP and found that only one insertion, one of 28 bp, had any activity. Screening hundreds of mutants of different, random, length, (from 6 bp to 240 bp), we conclude that the spacing requirement in DNA between two protein binding sites is rigid.

#### C. The physiology of CRP mutants.

Cells lacking CRP or cAMP exhibit phenotypes very different from wild type. Preliminary work from this laboratory has suggested that the translation of the galE gene is regulated by CRP. Expression of the galE gene is measured both by UDP-gal epimerase assay as well as by  $\beta$ -galactosidase assay in galE-lacZ translational fusions for CRP effects in vivo on galE expression. We find that there is increased translation of galE mRNA in a crp mutant. We are investigating the mechanism for this regulation. Possible explanations involve a CRP binding site overlapping the translational start site.

OxyR: We have currently started working on another gene regulatory protein OxyR that activates transcription of genes involved in turning on operons involved in  $O_2$  induced stress in cells. (1) OxyR protein is now being purified in large amounts to study it biochemically. (2) oxyR gene is being mutated to define the different functional domains of the protein.

#### Publications:

Ren YL, Garges S, Adhya S, Krakow J. Co-operative DNA binding of heterologous proteins: Evidence for contact between the cyclic AMP receptor protein and RNA polymerase, Proc Natl Acad Sci USA 1988;85:4138-42.

Garges S, and Adhya S. Cyclic AMP-induced conformational change of CRP: Intragenic suppressors of cyclic AMP-dependent crp mutants. J Bacteriol 1988;170:1417-22.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08751-09 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Regulation of the gal Operon of Escherichia coli

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

PI: S. Adhya Chief, Developmental Genetics Section LMB, NCI

Other: R. Haber	Microbiologist	LMB, NCI
J. Tokeson	Student Volunteer	LMB, NCI
N. Mandal	Visiting Scientist	LMB, NCI
C. Zwieb	Visiting Scientist	LMB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Developmental Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

2.5

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

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

B

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

Gal repressor protein negatively regulates the expression of the gal operon of E. coli. Repression requires binding of repressor to two spatially separated operator sites, O<sub>E</sub> and O<sub>I</sub>. We have previously shown that simple occupation of the two operators sites by repressor is not sufficient to achieve normal repression and proposed that an interaction between two operator bound dimeric repressors, giving rise to a DNA loop, is essential for repression.

The following experimental results strongly support our model of repression.

(1) We have shown that repressor dimers bound to O<sub>E</sub> and O<sub>I</sub> associate generating a DNA loop as studied by electron microscopy. (2) We have characterized a repressor mutant which binds to O<sub>E</sub> and O<sub>I</sub>, but does not tetramerize. This mutant protein when bound to DNA does not form a DNA loop as studied by electron microscopy. (3) The mutant repressor fails to repress the gal operon.

We have also discovered that in a cell devoid of Gal repressor, a derepressed gal operon can be further induced two- to three-fold by the addition of the inducer, D-fucose. In vivo operator titration assays suggest that the gal operon is subjected to a new negative control under conditions when the classical Gal repressor is removed from the cell.



### Major Findings:

We have previously shown in vivo that an interaction between the respective dimeric Gal repressor molecules bound to two gal operators,  $O_E$  and  $O_I$  is required to repress the gal operon. Mere occupation of the two sites by repressor is not sufficient for normal repression. We have also shown that Lac repressor can also repress the gal operon, provided that the two gal operator sequences are replaced by lac operator sequences. We are currently studying whether other sequence specific DNA binding proteins can mimic Gal and Lac repressor.

We have now shown that purified Lac repressor bound to the operators form the predicted DNA loop as observed under electron microscope. We have also characterized both in vivo and in vitro a mutant repressor which fails to repress the gal operon. We have proposed that the alteration has affected the protein's ability to tetramerize, which is required for repression. The prediction is confirmed by the following three observations:

1. The mutant protein binds to operators normally.
2. The mutant repressor when bound to DNA does not show DNA loop formation in electron microscope.
3. Whereas the wild-type repressor is a tetramer, the purified mutant repressor is a dimer.

We have shown that a complete deletion of the Gal repressor gene causes, as expected, high level expression of gal operon without inducer. However, the addition of inducer further elevates the level of expression by two- to three-fold. To investigate this effect, we have constructed numerous fusions of the gal operon to the gene encoding  $\beta$ -galactosidase. Analysis of both gene and operon fusions in the Gal-repressor deleted strains has shown that the second level of Gal repressor independent induction of the gal operon is at the level of transcription. In vivo competition binding experiments have suggested that this control is mediated by a negative regulator, which acts by binding to the same DNA segment that contains the gal operator promoter sequences. We are currently trying to identify the negative regulator by isolating mutants defective in this regulation.

### Publications:

Wartell RM, Adhya S. DNA conformational change in Gal repressor-operator complex: involvement of central G-C base pair(s) of dyad symmetry, Nucl Acids Res 1988;16:11531-41.

Haber R, Adhya S. Interaction of spatially separated protein-DNA complexes for control of gene expression: Operator conversions, Proc Natl Acad Sci USA 1988;85:9683-87.

Irani H, Musso R, Adhya S. Cyclic AMP dependent switch in initiation of transcription from the two promoters of the Escherichia coli gal operon: Identification and assay of 5' triphosphate ends of mRNA by GTP:RNA guanyltransferase, *J Bacteriol* 1989;171:1623-30.

Adhya S. Multipartite genetic control elements: communication by DNA loop, *Ann Rev Genetics* 1989, in press.

Zwieb C, Kim J, Adhya S. DNA bending by negative regulatory proteins: Gal and Lac repressors, *Genes and Development* 1989; in press.

Majumdar A, Adhya S. Effect of ethylation of operator-phosphates on Gal repressor binding: DNA contortion by repressor, *J Mol Biol*, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08752-09 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Mechanisms of Thyroid Hormone Action in Animal Cells

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

PI:	S.-y. Cheng	Research Chemist	LMB, NCI
Other:	C. Parkison	Chemist	LMB, NCI
	H. Kato	Visiting Fellow	LMB, NCI
	J. Lin	Visiting Fellow	LMB, NCI

## COOPERATING UNITS (if any)

P. McPhie Laboratory of Biochemistry and Metabolism NIDDKD

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Molecular Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

3.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

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

B

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

I. Molecular cloning of a human cytosolic thyroid hormone binding protein. A cDNA clone encoding a human cytosolic thyroid hormone binding protein (CTHBP) has been isolated. The sequence of CTHBP was found to be homologous to the subunit of rat pyruvate kinase, subtype M2 (PKM2). CTHBP, being a monomer, has 10-15% of the enzymatic activity of tetrameric PKM2. The tetrameric PKM2 lacks binding activity for 3,3',5-triiodo-L-thyronine ( $T_3$ ). Binding of CTHBP to  $T_3$  or its analogs resulted in the inhibition of pyruvate kinase activity. The apparent  $K_i$ 's of  $T_3$ , L-thyroxine, and D- $T_3$  are 30 nM, 100 nM, and 2  $\mu$ M, respectively. L-thyronine and reverse- $T_3$  had no effect. This order of activity correlates with the thermogenic effects reported for  $T_3$  and its analogs. Since PK is a key enzyme in regulating cellular ADP, ATP, and pyruvate, the present findings suggest that CTHBP may be involved in mediating some of the cellular metabolic effects induced by thyroid hormones.

II. Molecular mechanism of thyroid hormone nuclear receptor-thyroid hormone-DNA interaction.

Using a T7 expression system, large amounts of the human placental c-erbA protein (hc-erbA- $\beta$ ) were expressed and purified to apparent homogeneity. Analysis of the binding data indicated that the purified hc-erbA- $\beta$  binds to  $T_3$  with a  $K_a = 2.8 \times 10^9 M^{-1}$  and  $n = 0.3$  mole/mole. It binds to 3,3',5-triiodo-L-thyropropionic acid, 3,3',5-triiodo-L-thyroacetic acid, D- $T_3$ , L-thyroxine and 3',5',3-triiodo-L-thyronine with 475%, 120%, 39%, 7% and 0.1% respectively, of the activity of L- $T_3$ . This order of binding affinity of  $T_3$  analogs is similar to that reported for the  $T_3$  nuclear receptor identified from tissues or cultured cells. Furthermore, the purified hc-erbA- $\beta$  binds to the  $T_3$  response element of the rat growth hormone gene and the binding is hormone-independent. The availability of large amounts of active hc-erbA- $\beta$  should lead to the understanding of the mechanism on its transcriptional regulation by  $T_3$  at the molecular level.

Major Findings:

I. Molecular cloning of a human cytosolic thyroid hormone binding protein. A human cytosolic thyroid hormone binding protein (CTHBP) was purified to apparent homogeneity. The purified protein retains its binding activity and specificity. To study its cellular functions and regulation, the cDNA encoding CTHBP was isolated. Using two monoclonal antibodies against CTHBP, J11 and J12, we screened a  $\lambda$ gt11 expression library constructed from human thyroid carcinoma. Two clones, TC6 and TCB, with a length of 2.2 Kb and 2.3 Kb, respectively, were obtained. TCB is 79 bp longer than TC6 in the 3' untranslated region with a poly A tail which is absent in TC6. TCB contains a single open reading frame of 1593 nucleotides which encodes a polypeptide of 531 amino acids. A 29-amino acid partial sequence determined from the purified protein matches the deduced amino acid sequence. In vitro translation of mRNA prepared by transcription of the cDNA clone yielded a protein with the expected molecular weight of 58,000 and was recognized by J11 and J12. The size of mRNA in several human cultured cells hybridizable to TCB is ~2.4 Kb. These results indicate that TCB a full-length cDNA which encodes CTHBP.

The sequence of CTHBP was found to be very similar to that of the subunit of rat pyruvate kinase, subtype M2 (PKM2). The degree of homology between these two proteins is 91% at the level of nucleotide sequence and 97% at the level of amino acid sequence. CTHBP, being a monomer, has 10-15% enzymatic activity of PKM2. Its PK activity is inhibited by  $T_3$ , L-thyroxine and D- $T_3$  with an apparent  $K_i$  of 30 nM, 100 nM, and 2000 nM, respectively. L-thyronine and reverse- $T_3$  had no effect. This order of activity correlates with the thermogenic effects reported for  $T_3$  and its analogs. Since PK is a key enzyme in regulating cellular ADP, ATP, and pyruvate, the present findings suggest CTHBP may be involved in mediating the cellular metabolic effects induced by  $T_3$ . The role CTHBP plays in mediating  $T_3$ -induced thermogenesis and other metabolic effects is currently being studied.

II. Molecular mechanism of thyroid hormone nuclear receptor-thyroid hormone-DNA interaction.

Recent studies have shown that c-erbA protein is the thyroid hormone nuclear receptor (NR). To study structure-function relationships and to understand its three-dimensional structure by x-ray crystallography, large amounts of purified NR are needed. Since the successful purification of large amounts of purified NR from tissues has not been reported, we have adopted the recombinant DNA technique to produce it in Escherichia coli. An expression vector which contains a T7 phage promoter and the coding region of the human placental (c-erbA- $\beta$ ) was constructed. Upon induction with isopropyl-1-thio- $\beta$ -D-galactopyranoside, large amounts of hc-erbA- $\beta$  were expressed. The majority (70%) of the hc-erbA- $\beta$  expressed was concentrated in the insoluble inclusion bodies. The hc-erbA- $\beta$  was solubilized with guanidinium-HCl, renatured and purified. From one liter of E. coli culture, 1.5 - 2 mg of purified hc-erbA- $\beta$  was obtained. Analysis of the binding data indicated that the purified hc-erbA- $\beta$  protein binds to  $T_3$  with a  $K_a$  of  $5.9 \times 10^9 M^{-1}$ . It binds to 3'-isopropyl-3,5-diiodo-L-thyronine, 3,3',5-triiodo-L-thyropropionic acid, D- $T_3$ , L-thyroxine and 3',5',3-triiodo-L-thyronine with 312%, 195%, 31%, 8%, and



0.1%, respectively, of the activity of L-T<sub>3</sub>. This order of binding activity of the analogs is similar to that reported for the NR isolated from the nuclei of tissues of cultured cells. An oligomer containing the 29 bp sequence of the rat growth hormone gene response element (rGHRE), extending from -186 to -158 bp from the CAP site, was synthesized. The purified hc-erbA-β protein binds to the rGHRE, whereas only background binding was detected when a control oligomer with a sequence corresponding to the adenovirus 5 element was used. These results indicate that the purified hc-erbA-β is active. The availability of large amounts of the active NR should make it possible to study the molecular nature of receptor-hormone-DNA interaction by physio-chemical and biochemical methods.

#### Publications:

Fukuda T, Willingham MC, Cheng S-y. Antipeptide antibodies recognize c-erbA and a related protein in human A431 carcinoma cells, *Endocrinology* 1988; 123:2646-2652.

Obata T, Fukuda T, Willingham MC, Ling C-M, Cheng S-y. A cytoplasmic thyroid hormone binding protein: characterization using monoclonal antibodies, *Biochemistry* 1989;263:617-623.

Cheng S-y, McPhie P. Rhodamine b-labeled thyroid hormone forms high molecular weight aggregates in solution: A possible source of artifacts in binding experiments, *Anal Biochem* 1989;176:440-443.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08753-07 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Immunotoxin and Oncotoxin 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 NCI

Other: D. FitzGerald Microbiologist LMB, NCI  
 M.C. Willingham Chief, UCS LMB, NCI

## COOPERATING UNITS (if any)

Metabolism Branch, DCBD, NCI Laboratory of Viral Diseases, NIAID, NCI  
 Harvard Medical School Arthritis and Rheumatism Branch, NIAMS  
 Protein Design Labs

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Molecular Biology

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

10.0

## PROFESSIONAL:

7.0

## OTHER:

3.0

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Pseudomonas exotoxin (PE) or genetically modified forms of PE have been attached to monoclonal antibodies (mAbs) or growth factors to create cell-specific cytotoxic agents. Native PE attached to mAb OVB3 is cytotoxic to ovarian cancer cells, inhibiting the growth of ovarian cancer in mice, and is being currently tested in women with ovarian cancer. Mutant PE molecules in which domain I has been replaced by TGF $\alpha$ , IL2, IL4, or IL6 have been created by gene fusion and the chimeric proteins produced in *E. coli*. IL6-PE40 is cytotoxic to some myeloma cells. CD4 has also been fused to PE40 to create CD4-PE40, a chimeric toxin which kills cells infected with HIV. Such molecules may be useful in the elimination of tumor cells or other cells responsible for several human diseases.

Other Professional Personnel:

S. Adhya	Chief, Developmental Genetics Section, LMB, NCI
V. K. Chaudhary	Visiting Associate LMB, NCI
H. Lorberboum-Galski	Visiting Fellow LMB, NCI
J. Batra	Visiting Associate LMB, NCI
Y. Jinno	Visiting Fellow LMB, NCI
M. Ogata	Visiting Fellow LMB, NCI
C. Siegal	Biotechnology Fellow LMB, NCI
T. Waldmann	Medicine Branch, DCT NCI
R. Junghans	Medicine Branch, DCT NCI

Major Findings:

Pseudomonas toxin is a single chain bacterial toxin which has been genetically modified so that it selectively kills cancer cells and other cells which bear specific cell surface receptor molecules. Using an E. coli expression system, various mutant forms of PE have been prepared in order to determine which portions of the molecule are required for optimal cell killing activity. These studies have shown that deletion of all of domain I (amino acid 1-253) yields a molecule (PE40) which has very low cytotoxic activity because it no longer binds to the PE receptor that is present on all cells. When domain I is replaced with different cell targeting molecules, novel chimeric toxins are created which kill specific types of cells. These include TGF $\alpha$ -PE40 which kills many cancer cells with EGF receptors, IL2-PE40 which kills cells including leukemia cells with IL2 receptors, IL4-PE40 which kills cells with IL4 receptors, and IL6-PE40 which kills certain myeloma cells. To kill HIV infected cells, domain I was replaced with CD4 to create a hybrid molecule (CD4-PE40) that kills HIV infected cells because it binds to the envelope protein gp120 present on the surface of cells producing the AIDS virus. Methods have been developed which allow the preparation of large amounts of these proteins in pure form.

IL2-PE40 was found to be cytotoxic to cells bearing either the p55 of the p75 subunit of the IL2 receptor but much more cytotoxic to cells with both receptor forms reflecting the fact that the high affinity receptor is rapidly internalized. Activation of mouse T cells by Concanavalin A or by mixed leukocyte culture also rendered the T cells IL2-PE40 sensitive due to expression of IL2-receptors. Several models of animal diseases known to be associated with enhanced IL2 receptor expression were used to evaluate possible therapeutic effects of IL2-PE40. IL2-PE40 treatment was found to tolerize mice so that they would accept cardiac allografts and to suppress the adjuvant induced arthritis in rats. In both instances, it is assumed that the agent works by selectively eliminating clones of cytotoxic T cells.

In patients with AIDS, HIV replicates in T cells ultimately leading to the destruction of CD4 positive cells. In a therapy designed to eliminate infected cells and arrest viral spread, a portion of the CD4 molecule was fused to PE40 to create CD4(178)-PE40; this protein binds to and kills HIV infected cells. CD4-PE40 not only kills cells infected with different strains of HIV, but it

also suppresses the spread of HIV in mixed cultures of infected and uninfected cells. These properties are essential if the agent is to be effective in the treatment of patients with AIDS. CD4-PE40 is now undergoing preclinical studies.

Active immunotoxins have been created by chemically coupling PE or PE40 to antibodies. Using gene splicing techniques, a novel single chain immunotoxin has been created in which the COOH end of the V<sub>H</sub> domain of an antibody is attached to the amino end of a V<sub>L</sub> domain by a linker peptide and the carboxyl end of the V<sub>L</sub> domain is attached to PE40. Using this strategy, an anti-Tac(Fv)-PE40 molecule was made in E. coli and shown to be cytotoxic to cells bearing human IL2 receptors.

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Chaudhary VK, Xu Y-h, FitzGerald D, Adhya S, Pastan I. Role of domain II of Pseudomonas exotoxin in the secretion of proteins into the periplasm and medium by E. coli, Proc Natl Acad Sci USA 1988;85:2939-43.

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Siegall CB, Chaudhary VK, FitzGerald DJ, Pastan I. Cytotoxic activity of an interleukin 6-pseudomonas exotoxin fusion protein on myeloma cells, Proc Natl Acad Sci USA 1988;85:9738-42.

FitzGerald DJ, Willingham MC, Pastan I. Pseudomonas exotoxin--Immunotoxins. In: Frankel A, ed. Immunotoxins. Norwell MA: Kluwer Academic Publishers. 1989;161-173

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Lorberboun-Galski H, Barrett LV, Kirkman RL, Ogata M, Willingham MC, FitzGerald DJ, Pastan I. Cardiac allograft survival in mice treated with IL2-PE40, Proc Natl Acad Sci USA 1989;86:1008-1012.

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Pearson JW, FitzGerald DJP, Willingham MC, Wilttrout RH, Pastan I, Longo DL. Chemo-immunotoxin therapy against a human colon tumor (HT-29) xenografted into nude mice, Cancer Res 1989; in press.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08754-06 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells

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

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

## COOPERATING UNITS (if any)

Division of Cancer Therapy, NCI

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Molecular Cell Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

12.0

## PROFESSIONAL:

12.0

## 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.)

Resistance to multiple drugs is a major impediment to the successful chemotherapy of human cancers. One mechanism of multidrug-resistance is the expression of a 170,000 dalton energy-dependent drug efflux pump (P-glycoprotein) which confers resistance to colchicine, adriamycin, vincristine, vinblastine, puromycin, and actinomycin D. ATP-dependent transport of vinblastine has been demonstrated using membrane vesicles from MDR cells. This transport is inhibited by vincristine, daunomycin, actinomycin D and colchicine as well as verapamil, quinidine and other drugs which reverse multidrug-resistance. Expression vectors into which the MDR1 cDNA is cloned confer the complete MDR phenotype on drug-sensitive cells, as does a retrovirus carrying the MDR1 gene. A chimeric P-glycoprotein, in which a functional adenosine deaminase (ADA) molecule is attached to the C-terminus, has been constructed. When introduced into dog kidney epithelial (MDCK) cells, the MDR1 cDNA is expressed on the apical surface of the epithelia, and allows transport of drugs such as vinblastine, daunomycin, actinomycin D and verapamil. Transgenic mice carrying the human MDR1 gene expressed in bone marrow, are resistant to daunomycin-induced leukopenia. Expression of MDR1 RNA and P-glycoprotein normally occurs in kidney, liver, colon, and adrenal and in tumors derived from these tissues which are intrinsically resistant to chemotherapy as well as several other tumors. Acquired drug-resistance in leukemia, lymphoma, neuroblastoma, rhabdomyosarcoma, pheochromocytoma, breast and ovarian cancer may be associated with increased MDR1 RNA levels.

Other Professional Personnel:

M. C. Willingham	Chief, USC	LMB, NCI
C. Cardarelli	Research Biologist	LMB, NCI
S. Kane	Guest Researcher	LMB, NCI
H. Galski	Visiting Fellow	LMB, NCI
L. Goldstein	Medical Staff Fellow	LMB, NCI
E. Bruggemann	Guest Researcher	LMB, NCI
U. Germann	Guest Researcher	LMB, NCI
S. Currier	IRTA Fellow	LMB, NCI
P. Marino	Guest Researcher	LMB, NCI
F. Thiebaut	Visiting Fellow	LMB, NCI
S. Tanaka	Visiting Fellow	LMB, NCI
M. Horio	Visiting Fellow	LMB, NCI
K.-V. Chin	Visiting Fellow	LMB, NCI
R. Padmanabhan	Research Chemist	LMB, NCI
G. T. Merlino	Senior Staff Fellow	LMB, NCI
P. Schonlein	IRTA Fellow	LMB, NCI

Major Findings:

1. Membrane vesicles prepared from MDR cell lines transport vinblastine in the presence of ATP and an ATP regenerating system. This transport is inhibited by the drugs vinblastine, daunomycin, actinomycin D and colchicine, and by several drugs which reverse the MDR phenotype including verapamil, quinidine, diltiazem and nifedipine.
2. Transgenic mice carrying  $\beta$ AP-MDR, MT-MDR, and SV-MDR expression vectors have been constructed. One line of  $\beta$ AP-MDR mice has been studied in detail. Expression of human MDR1 mRNA has been found in several tissues of these transgenic mice including bone marrow and spleen. These mice are resistant to daunomycin-induced leukopenia.
3. Normal kidney, liver, colon, small intestine and adrenal express P-glycoprotein. In kidney, liver, colon and small intestine, P-glycoprotein is located on the apical (luminal) surfaces of transporting epithelia, consistent with a transport function for this protein. Specialized capillary endothelial cells of the brain and testes also express P-glycoprotein.
4. MDCK cells form epithelia which transport vinblastine, vincristine, daunomycin, actinomycin D, and verapamil from their basal to their apical surfaces. MDCK cells infected with the human MDR1 gene show increased transport of these agents. These results support the model of the P-glycoprotein as a multidrug transporter capable of carrying out transepithelial transport.
5. The MDR1 gene is expressed in untreated human tumors including many tumors derived from the colon, kidney, adrenal gland, liver and pancreas in addition to chronic myelogenous leukemia in blast crisis. Occasionally elevated

expression is found in other untreated tumors such as neuroblastoma, acute lymphocytic leukemia in adults, acute non-lymphocytic leukemia in adults, carcinoid tumors, indolent non-Hodgkins lymphoma and in cell lines of non-small cell carcinoma of the lung with neuroendocrine properties. Increased expression was detected in some tumors at relapse after chemotherapy including acute lymphoblastic leukemia of childhood, neuroblastoma, rhabdomyosarcoma, pheochromocytoma and nodular poorly differentiated lymphoma. Other untreated tumors usually express poorly differentiated lymphoma. Other untreated tumors usually express undetectable or low levels of MDR1 RNA; these include head and neck cancer, Wilm's tumor, esophageal cancer, breast cancer, ovarian cancer, small cell carcinoma of the lung, and other non-small cell carcinomas of the lung, the evaluation of MDR1 expression in both intrinsic and acquired drug resistance may prove to be a valuable tool in the design of chemotherapeutic protocols.

6. MDR1 RNA can be detected semiquantitatively by in situ hybridization analysis in MDR tissue culture cells. Loss of resistance occurs by segregation of amplified genes in a heterogeneous manner with small "islands" of MDR RNA positive cells remaining. In situ hybridization shows some promise as a technique for clinical detection of MDR1 RNA positive tumors in which expression of RNA might be heterogeneous.

7. A chimeric protein consisting of P-glycoprotein with adenosine deaminase (ADA) attached to its C-terminus has been created. The cDNA for this chimera when expressed in drug-sensitive NIH 3T3 cells under control of the HaMSV promoter, renders cells multidrug-resistant and increases ADA activity. Selection in increasing concentrations of colchicine results in increased expression of the multidrug transporter and increased ADA activity. This chimeric protein is potentially useful for gene therapy of ADA deficiency.

8. Several polyclonal antibodies have been prepared against synthetic peptides and fragments of P-glycoprotein expressed in E. coli. These antisera are useful reagents for immunoprecipitations and Western blots, and their specificity for specific regions of P-glycoprotein allows their use to dissect the function of different regions of the molecule.

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Roninson IB, Shen D w, Chin JE, Choi K, Fojo A, Soffir R, Richert N, Gros P, Housman DE, Gottesman MM, Pastan, I. Multidrug resistance in human cells: The role of the mdr1 gene. In: ICN-UCLA Symposium, Cellular and molecular biology of tumors and potential clinical applications. New York: Alan R Liss, Inc, 1988;287-296

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08756-02 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

The Transgenic Mouse as a Model System to Study Gene Function and Regulation

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

PI:	G. T. Merlino	Expert	LMB, NCI
Other:	I. Pastan	Chief, Laboratory of Molecular Biology	LMB, NCI
	M. M. Gottesman	Chief, Molecular Cell Genetics Section	LMB, NCI
	P. Marino	Guest Researcher	LMB, NCI
	H. Galski	Visiting Fellow	LMB, NCI
	C. Jhappan	Visiting Fellow	LMB, NCI
	C. Butler	Biologist	LMB, NCI

## COOPERATING UNITS (# any)

D. Lowy, Chief, Laboratory of Cellular Oncology DCSD, NCI

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Molecular Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

3.5

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

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

B

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

The production of transgenic mice constitutes a powerful approach to the study of gene function and regulation. We are using this new technology to investigate two important aspects of cancer progression: the ability of malignant cells to escape destruction by chemotherapy, and the role of oncogenes and growth factors in the development of the transformed phenotype.

Transgenic mice which contain a human gene (MDR1) encoding a multidrug transporter (P-glycoprotein) capable of conferring multidrug resistance have been generated. Expression of the human MDR1 gene was detected in the normally drug-sensitive bone marrow of several lines of transgenic mice. The transgenic bone marrow became resistant to the cytotoxic effects of the drug daunomycin. Since bone marrow suppression is a major drawback to the use of chemotherapy, these transgenic mice will be used as a model to determine if higher doses of drugs can cure previously unresponsive cancers.

The epidermal growth factor (EGF) receptor serves as an intermediary to the potent mitogens EGF and transforming growth factor alpha (TGF $\alpha$ ), and is the cellular homolog of the avian erythroblastosis virus erbB oncogene product. To elucidate the role that EGF receptor and its ligands play in growth control and transformation, we made mice bearing either the human EGF receptor gene or the human TGF $\alpha$  gene. In both cases the transgene was expressed in a wide variety of mouse tissues. Studies are underway to determine the effects of overexpression and inappropriate expression on the developing and adult mouse.

Major Findings:

Transgenic mice have been made by microinjecting a variety of purified DNA fragments into one-cell mouse embryos (either C57BL/6 X SJL hybrid mice or CD-1 outbred mice). The resulting embryos were reimplanted into pseudopregnant CD-1 foster mothers. Tail DNA from two week old offspring was analyzed for foreign DNA integration. Mice containing the transgene were expanded and lines established.

A human gene has been isolated (MDR1) that encodes a 170 kDa glycoprotein (P-glycoprotein) capable of conferring multidrug resistance to animal cells (see Annual Report Number Z01 CB 08754-06 for details). Several lines of transgenic mice were generated carrying the MDR1 cDNA driven by the chicken  $\beta$ -actin gene promoter. Transgenic RNA and protein have been detected in the bone marrow, which is normally extremely sensitive to chemotherapeutic agents. By injecting one such agent, daunomycin, into normal and transgenic mice we have been able to demonstrate that the transgenic bone marrow becomes drug resistant, and bone marrow suppression is greatly ameliorated. These mice are being used as a model to test the efficacy of using higher doses of chemotherapy to cure previously unresponsive cancers.

In collaboration with D. Lowy, we have found that mouse NIH3T3 cells infected with a retrovirus carrying the EGF receptor coding region overexpress the receptor, undergo EGF-dependent transformation in tissue culture, and form large tumors in nude mice. We concluded that overexpression of the EGF receptor or its ligand can contribute to the appearance of the transformed phenotype in cells in culture. To examine the effects of overexpression in vivo, we made transgenic mice carrying human cDNAs encoding either the EGF receptor or its ligand, TGF $\alpha$ . Mice containing the EGF receptor gene driven by the chicken  $\beta$  actin promoter expressed receptor RNA in a wide variety of mouse tissues. Another line was generated in which the TGF $\alpha$  gene was driven by the mouse MT-1 metallothionein gene promoter. In this line, inducible TGF $\alpha$  expression was also detected in a number of tissues, as well as the blood and urine. Preliminary results suggest that several mouse tissues are sensitive to the presence of these transgene products. We are using these mice as models to test the consequences of overexpression of EGF receptor and/or its ligand in tissues of the developing and adult mouse.

In a related project intended to determine the transcriptional mechanism by which cellular oncogenes such as the EGF receptor gene are regulated, the receptor gene promoter was isolated and characterized by protein-binding assays and mutagenesis analysis (see Annual Report Number Z01 CB 08000-19 for details). For example, I have identified a protein binding site that is both necessary for optimal expression in cultured cells and sensitive to single-stranded nucleases. Nuclease sensitivity has been shown to be associated with altered DNA structure and activity. To validate transfection assays and to determine how the promoter functions in vivo, promoter fragments were placed upstream of the chloramphenicol acetyltransferase (CAT) gene, and the resulting hybrid DNAs used to make transgenic mice. A number of such transgenic lines have now been made, and are presently being analyzed to determine the pattern of expression of this proto-oncogene promoter.

Publications:

Kageyama R, Merlino GT, Pastan I. A transcription factor active on the epidermal growth factor receptor gene, Proc Natl Acad Sci USA 1988;85:5016-5020.

Johnson AC, Jinno Y, Merlino GT. Modulation of EGF receptor proto-oncogene transcription by a promoter site sensitive to S1 nuclease, Mol Cell Biol 1988; 8:4174-4184.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08757-02 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Development of Immunotoxins for Cancer

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

PI:	D. J. FitzGerald	Microbiologist	LMB, NCI
Other:	I. Pastan	Chief, Laboratory of Molecular Biology	NCI
	M. C. Willingham	Chief, UCS	LMB, NCI
	M. Ogata	Visiting Fellow	LMB, NCI
	J. Batra	Visiting Associate	LMB, NCI
	T. Idziorek	Visiting Fellow	LMB, NCI

## COOPERATING UNITS (if any)

J. Pearson, FCRF	J. Shiloach,	BioPilot Plant, LCDB, NIDDK
D. Longo, FCRF	R. Heinrichson	Upjohn Company

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Ultrastructural Cytochemistry Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

3.0

## OTHER:

1.0

## CHECK APPROPRIATE BOXES)

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

B

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

To study the structure-function relationships of *Pseudomonas* exotoxin (PE):

1) A series of ten monoclonal antibodies were selected. Eight of these were further characterized and shown to react with either domains II, Ib, or III of PE. Only two of the eight antibodies reacted with native PE. The other six were presumed to react with cryptic epitopes, since they became exposed when PE was immobilized on nitrocellulose or denatured with urea and DTT. 2) The hydrophobic properties of PE were explored. Full length PE or domains I, II, and III were each shown to bind more detergent at pH 4.5 than at pH 7.0. 3) PE has an amino acid sequence resembling a consensus cleavage site for HIV protease. While PE was not degraded by this protease, PE40 was susceptible and was cleaved between residues 389-390.

PE-related immunotoxins: 1) Large quantities of LysPE40 are required for immunotoxin production. A method was developed to produce gram quantities of LysPE40. BL21 (DE3) was transformed with the plasmid pVC85 and grown to a very high density (absorbance of 40 units/ml at 650 nm was typical). The conditions required to achieve this level of growth included supplying gaseous oxygen, controlling pH by the addition of ammonium hydroxide and maintaining adequate glucose. Crude LysPE40 was then purified using Q Sepharose, QMA, Mono S and gel filtration chromatography. 2) LysPE40 immunotoxins were made with the following antibodies: 225 (anti-EGF R), HB21 (anti-TF R), antiTac (anti-IL2R), OVB3 (anti-ovarian Ca), anti-CD3, anti-CD5, and anti-CD33. 3) OVB3-LysPE40 is poorly cytotoxic, primarily because the OVB3 antigen is not well internalized. To increase the rate of endocytosis, and thereby enhance cytotoxic activity, transferrin was chemically coupled to OVB3-LysPE40. This produced a conjugate that is about ten-fold more active than OVB3-LysPE40. 4) Other methods of enhancing immunotoxin activity used verapamil analogs.

Major Findings:PE Structure-Function

PE is a proenzyme and must be unfolded, or activated by some other means, to express full ADP-ribosylating activity. PE40 lacks the toxin's binding domain and is also characterized by having full ADP-ribosylating activity. Thus, no unfolding of PE40 is necessary to obtain full ADP-ribosylating activity. To study how PE is unfolded, a series of monoclonal antibodies was isolated to PE40. Ten murine monoclonal antibodies (all IgGs) that reacted with PE were initially selected and eight of these were characterized further. Two of these eight appeared to bind epitopes that are exposed on soluble forms of PE and PE40. The other six antibodies reacted well with PE40, but PE had to be denatured or immobilized on nitrocellulose before reactivity could be demonstrated. A strong correlation was observed between conditions known to activate ADP-ribosylating activity and conditions needed to expose the cryptic epitopes. These antibodies will be used to study the activation and processing of PE within cells.

PE enters cells via coated pits and endocytic vesicles of low pH. Exposure to low pH is thought to be necessary before PE, or an active fragment, can gain access to the cell cytoplasm. Because of this, evidence was sought for low pH-dependent hydrophobic changes within PE or its various domains. Native PE, domain I, domains II-III, and domain III were incubated with Triton X-114 at pHs between 7.0 and 3.0. At pH 4.5 and below, there was a significant increase in the hydrophobicity of all these proteins. Thus, at low pH, PE becomes "globally" hydrophobic.

PE40 as a Substrate for HIV Protease

The protease encoded by HIV is necessary to produce infectious virions. However, the mechanism by which it releases itself and other proteins from the HIV polyprotein is currently poorly understood. The HIV protease is thought to act by cleaving multidomain proteins in between their structural domains. Since the three-dimensional structure of PE is known and consists of three structural domains, it and PE40 were tested as potential substrates. A proposed consensus cleavage site was noted between domains II and III at residues 394-395. When mixed with purified HIV protease, PE was not cleaved. However, PE40 was cleaved and at only one site. Unexpectedly, this was between residue 389 and 390 and not 394-395. CD4-PE40 was also cleaved at the 389-390 site.

lysPE40

LysPE40-immunotoxins were made with the following antibodies: 225 (anti-EGF receptor), HB21 (anti-TF receptor), antiTac (anti-IL2 receptor), OVB3 (anti-Ovarian Ca), anti-CD3, anti-CD5, and anti-CD33. OVB3-lysPE40 was poorly cytotoxic, primarily because the OVB3 antigen is not internalized very efficiently. Determinations made by indirect immunofluorescence showed that neither the OVB3 antibody nor OVB3-lysPE40 were internalized after incubations for one hour at 37°C. In contrast, OVB3-PE was internalized quite well. Thus, some feature of the PE binding domain mediates the internalization of the OVB3 antibody. To increase the rate of endocytosis and, thereby, enhance cytotoxic activity, transferrin was chemically coupled to OVB3-lysPE40. This produced a conjugate that was about ten-fold more active than OVB3-lysPE40. This type of conjugate was made in one of two ways. In the first instance, a transferrin-lysPE40 conjugate was made and this was coupled to the OVB3

antibody (O-TlysPE40), and in the second, transferrin was coupled to OVB3-lysPE40 (T-OlysPE40). Both types of conjugate were cytotoxic and this activity was displaced by either excess transferrin or excess OVB3.

Other methods of increasing immunotoxin activity were also explored. Various verapamil analogs were shown to enhance the activity of both PE-immunotoxins and ricin A chain immunotoxins.

#### Publications:

- Kondo T, FitzGerald D, Chaudhary V, Adhya S, Pastan I. Activity of immunotoxins constructed with modified pseudomonas exotoxin A lacking the cell recognition domain, *J Biol Chem* 1988;263:9470-5.
- Pirker R, FitzGerald DJP, Willingham MC, Pastan I. Enhancement of the activity of immunotoxins made with either ricin A chain or Pseudomonas exotoxin, *Cancer Res* 1988;48:3919-23.
- Jinno Y, Chaudhary VK, Kondo T, Adhya S, FitzGerald DJ, Pastan I. Mutational analysis of domain I of pseudomonas exotoxin: Mutations in domain I of pseudomonas exotoxin that reduce cell binding and animal toxicity, *J Biol Chem* 1988;263:13203-7.
- FitzGerald D, Pastan I. Pseudomonas exotoxin and derived conjugates: Interactions with mammalian cells. In: Steer CJ, Hanover J, eds. *Intracellular Trafficking of Proteins*. England: Cambridge University Press, 1989, in press.
- Pearson JW, FitzGerald DJP, Willingham MC, Wiltrout RH, Pastan I, Longo DL. Chemo-immunotoxin therapy against a human colon tumor (HT-29) xenografted into nude mice, *Cancer Res* 1989; in press.
- FitzGerald D, Pastan I. Targeted toxin therapy for the treatment of cancer, *J Natl Cancer Inst* 1989; in press.

## SUMMARY

Annual report of the Laboratory of Biochemistry, National Cancer Institute  
October 1, 1988 to September 30, 1989

As in the past, the diverse research projects of the Laboratory of Biochemistry reflect the organization of the laboratory into many independent groups with different interests and expertise. However, these differences have become less evident since several groups interested in the control of gene expression have turned their attention to the isolation and characterization of trans-acting regulatory proteins and groups studying cellular regulation or protein structure have been involved in cloning and expressing proteins in bacterial and eukaryotic cells. This transition has been facilitated by the presence in the laboratory of complementary expertise and has increased the interactions among the members of the laboratory. The laboratory staff welcome the participation of Shelby Berger and her colleagues, who joined the Laboratory this spring, to our weekly informal seminars. Shelby brings to the laboratory a new interesting problem on the regulation of cell division, valuable experience in protein chemistry and molecular biology and a critical mind.

### THE CONTROL OF GENE EXPRESSION.

The regulation of gene expression remains the focus of interest for many members of the Laboratory. During the last year major progress has been made in the isolation and characterization of several trans-acting regulatory proteins.

Carl Wu's group has continued to study sequence-specific DNA-binding proteins in Drosophila, focusing on those binding at the heat shock genes and the segmentation gene fushi tarazu, ftz. A major achievement this year was cloning of heat shock gene transcriptional activator, or Heat Shock Factor, HSF. Another important finding was the demonstration that HSF, which exists in an inactive conformation, can be activated in vitro by exposure to heat, low pH or an antibody raised against the active form of HSF suggesting that a conformational change may be responsible for heat activation. It is hoped that elucidation of the nature of this structural change will provide insight into the mechanism by which this key signal transducer senses environmental stress and transmits the signal to the transcriptional machinery. The heat shock gene will be used as a model to study the cooperative or competitive interactions between nucleosomes and transcription factors by in vitro reconstitution experiments. These studies may help to clarify the role of histone proteins in controlling gene activity in higher cells. The second project under study by this group deals with the regulation of gene expression allowing temporal and spatial expression of the ftz gene during Drosophila development. A DNA-binding factor that interacts with the ftz gene, ftz1, has been isolated. The function of ftz1 has been studied in vivo in transgenic flies and shown to be involved as a positive transcription factor in specific segments of the early embryo.

Another aspect of regulation of gene expression during development is being studied in Bruce Paterson's laboratory. This group is focusing on the regulation of genes associated with muscle development and on characterization of the genes involved in myogenic determination. They previously identified cis-acting elements involved in the developmentally regulated expression of muscle specific genes. They have now shown that the regulated expression of a-



actin and some myosin light chain genes in muscle is the result of a complex interplay of negative and positive regulatory proteins binding at the same DNA regulatory regions. The suppression of the  $\beta$ -actin gene in differentiated muscle is controlled by a cis-acting element in the 3' noncoding region of the gene. This element imparts the  $\beta$ -actin regulatory pattern when inserted, in either orientation, within the transcriptional unit of any gene expressed in a muscle cell background. A related and exciting development is the isolation of the CMD1 gene which is involved in the determination of the myogenic phenotype. BUdR, a thymidine analog that inhibits myogenesis, also inhibits the expression of CMD1 and may explain how the drug blocks differentiation. Expression of CMD1 in BUdR-treated cells will activate a cotransfected muscle specific promoter and, in some cases, the endogenous muscle genes. The isolation and mode of action of the CMD1 gene product has become a major concern for the group led by Bruce Paterson.

This year Dean Hamer and his colleagues have made an important breakthrough in their understanding of how copper regulates metallothionein gene transcription in yeast. They discovered that copper binds directly to a bifunctional transcription factor, ACE1, and alters the conformation of a "DNA-binding domain" such that it can bind to DNA. Once the factor has bound to the metallothionein gene control sequences, a separate "activation domain" interacts with general transcription factors and thereby accelerates the rate of transcription initiation. The copper and DNA binding domain of ACE1 represents a new type of DNA recognition motif which they dub the "copper fist". In higher eukaryotes, good progress has been made on isolating a mouse metallothionein gene regulatory factor, and the gene for an unusual zinc finger protein, also involved in the regulation of expression of the metallothionein gene, has been cloned and characterized.

The role of DNA methylation in the modulation of gene expression has become the main focus of interest in Dr. Kuff's laboratory. For many years his group has been studying the regulation of an envelope-deficient mouse retrovirus, the intracisternal A-particle (IAP). IAPs are encoded by members of a multicopy family of endogenous proviral elements. IAP elements are transposable in the mouse genome and are multigenic. They are expressed in mouse embryos, some normal adult tissues, and many transformed mouse cells and represent a good model system to study gene regulation. Expression requires demethylation of sequences within the IAP 5' LTR along with a constellation of trans-acting cellular factors. The genetic basis for selective demethylation of IAP provirus is being investigated in normal mouse lymphocytes and pancreatic b cells, while relevant transcription factors are being studied in nuclear extracts of various transformed rodent and primate cells.

## PROTEINS AND CONTROL OF CELLULAR PROCESSES

The mechanism of stimulus response coupling mediated by  $\text{Ca}^{2+}$  and calmodulin remains the focus of interest in Claude Klee's laboratory. In collaboration with Katheen Beckingham (Rice University) they have used calmodulin mutants deficient in  $\text{Ca}^{2+}$  binding at either the low or high affinity  $\text{Ca}^{2+}$ -binding sites to study the cooperative binding of  $\text{Ca}^{2+}$  to the two halves of

calmodulin. The same mutants were used to demonstrate that  $\text{Ca}^{2+}$  binding to all four sites of calmodulin plays a role in the activation of calcineurin, a calmodulin-stimulated protein phosphatase. A major achievement during the last year was the isolation and sequencing of two different cDNA clones of the catalytic subunit of calcineurin by D. Guerini. The deduced amino acid sequences of the two proteins have allowed the characterization of the calmodulin-binding domain, the identification of a novel structural domain needed to maintain enzyme activity and revealed the extensive similarity of its catalytic domain with the catalytic subunits of protein phosphatases 1 and 2a thereby defining a family of related protein phosphatases.

The group led by Paul Wagner has started a new project concerned with the regulation of catecholamine secretion by chromaffin cells. They have demonstrated that  $\text{Ca}^{2+}$ -dependent norepinephrine secretion by permeabilized PC12 cells occurs in two steps: A first  $\text{Ca}^{2+}$ -dependent phosphorylation, rendered practically irreversible by the use of ATP $\gamma$ S, is followed by a  $\text{Ca}^{2+}$ -independent but still ATP-dependent step. These studies provide preliminary evidence that phosphorylation regulates secretion. An alternative mechanism for regulation of secretion involving GTP-binding proteins is also under study.

Shelby Berger and her colleagues are studying the role of prothymosin  $\alpha$  in cell division. The human prothymosin  $\alpha$  gene family has been cloned, and sequenced. It consists of one functional gene, and five processed pseudogenes. The gene gives rise to two mRNAs by a novel form of alternate splicing. Functional studies on prothymosin  $\alpha$  mRNAs, synthetic and natural proteins, and transfected cDNA and genes are not consistent with the extracellular role for thymosin  $\alpha$  proposed by others. Preliminary evidence suggests that prothymosin  $\alpha$  is required intracellularly for cell division. This group is also investigating the mechanism of action of the ribonuclease H of two viral reverse transcriptases. Contrary to previously held views these ribonucleases are endonucleases. They have identified a specific inhibitor of the viral enzyme.

Dr. Beverly Peterkofsky has continued to make steady progress in her study of the regulation of the synthesis of the extracellular matrix components, collagen and proteoglycan. Her previous proposal that the decreased synthesis of these matrix components in ascorbate-deficient guinea pigs is not related to the role of ascorbate in the hydroxylation of proline in collagen but to changes in circulating factors induced by fasting is supported by the demonstration that sera from scorbutic and fasting animals (ascorbate supplemented) have reduced levels of insulin-like growth factor (IGF-I) and also contain an inhibitor of IGF-I. This inhibitor has now been shown to be a low molecular weight IGF-I-binding protein. As part of their study of the alteration of collagen synthesis in transformed cells Dr. Peterkofsky and her colleagues have shown that the defect in normal collagen synthesis in transformed cells is due to the loss of expression of the gene for the precursor of one of the two subunits of the type I collagen, accompanied by a post-translational modification of the precursor of the other subunit.

In order to study receptor-mediated activation of T cells, and the role of T cells in tumor rejection, Michael Mage and his associates had developed polyvalent, multimolecular conjugates of the major histocompatibility complex (MHC) and soluble polymers, "polygens". A

modification of this method based on streptavidin-polymer complexes (PASA) allows them to form conjugates of PASA with biotinylated antibodies. One of their goals is to use these streptavidin-polymer complexes to label tumor cells with MHC antigens with the hope that they will be recognized by killer cells and thereby facilitate tumor rejection. They intend to apply a similar approach to the development of immunotoxins .

Warren Evans and his colleagues are studying the end-stage maturation of granulocytic leukocytes in order to understand defects in this maturation process in human leukemia. Using a cell culture assay, developed in their laboratory, to quantitate conversion of myelocyte precursors to mature granulocytes they have demonstrated the presence of a granulocyte maturation factor (GMF) in human serum that is absolutely required for maturation of human granulocytes. A 250-fold purification of GMF has been achieved.

### DNA REPLICATION.

Three independent groups are running active programs devoted to the study of prokaryotic and eukaryotic DNA replication.

Several lines of inquiry have been pursued in studies of P1 plasmid maintenance by Drs. Michael Yarmolinsky and Dhruva Chatteraj of the Microbial Genetics and Biochemistry Section. Biochemical studies have shown the importance of steric factors in (1) the control of plasmid replication by DNA sites that bind the plasmid initiator protein and (2) the efficient functioning of the P1 centromere. Genetics studies have revealed the involvement of several heat shock proteins in plasmid replication. Physiological studies of the effects of interference with plasmid replication functions have led to a detailed analysis of the P1 lytic replicon and of P1 genes involved in bacterial addiction to the presence of the plasmid.

Dr. Michael Lichten, a member of the Microbial Genetics and Biochemistry Section, is studying the mechanism and regulation of meiotic recombination in the yeast Saccharomyces cerevisiae. Techniques to detect heteroduplex DNA have been refined and are now being used to determine the specificity of base-pair mismatch correction, the polarity of strand transfer during meiosis, and the timing of formation of heteroduplex DNA during meiosis. The role played by meiotic transcription and chromatin structure in determining the level of meiotic recombination in a particular region is under study. Finally, a search for sequences which modulate the level of meiotic recombination in their vicinity has been initiated.

The earlier success in cloning a cDNA of human  $\beta$ -DNA polymerase by Samuel Wilson and his collaborators has been followed by the characterization of the core promoter for the human gene and the identification of a palindromic sequence GTGACGTCAC as a critical promoter element. A transcription factor that binds specifically to the palindromic sequence was purified and characterized. The preferential expression of this enzyme in testis and its induction by treatment with mutagens may shed additional light on its physiological role. The rat DNA polymerase  $\beta$  and the A1 hnRNP single strand-binding protein were over expressed in E. coli and their domain structure studied by limited proteolysis. The nucleic acid binding properties of the



individual domains are under study. The determination of the kinetic parameters of the polymerization reaction catalyzed by HIV reverse transcriptase carried out by Sam Wilson and his colleagues is a first step in their attempts to identify specific inhibitors of the enzyme.

## THE ORGANIZATION OF THE HUMAN GENOME.

The panel of human-rodent somatic cell hybrids previously isolated and characterized in Wesley McBride's laboratory continues to be an asset for many investigators at NIH and elsewhere wishing to determine the chromosomal localization of human genes. About 20-25 genes have been mapped by this procedure during the past year. They include lymphokines, neoplastic breakpoints, and genes involved in DNA repair, cell adhesion, regulation of cell proliferation, transcriptional regulation, and cellular regulatory pathways. The gene size, organization and copy number at each locus was determined. Related observations included the localization of some of these genes near chromosomal breakpoints in neoplasms and the variable amplification of some loci in different individuals. Several have been more precisely localized within chromosomes by *in situ* hybridization of metaphase chromosome spreads. Southern blots of DNA restriction digests of DNAs from 40 large kindreds have been prepared and used for regional gene localization by linkage analysis using probes which detect restriction fragment length polymorphism. About 20 genes on 7 different human chromosomes have been mapped by this method during the past year; the role of several of these candidate genes in specific hereditary diseases will be evaluated.

Observations made this year in Dr. Maxine Singer's group extend earlier work on the expression of LINE-1 moveable elements in the human genome. These workers demonstrated that DNA sequences essential to the promoter activity of the 900 bp long 5'-leader segment of human LINE-1 elements lie within the first 100 bp at the 5' end. Moreover, LINE-1 promoter activity is about 100 fold higher in human teratocarcinoma cells than in HeLa cells. This finding is consistent with the earlier observation by this group that full length cytoplasmic polyA<sup>+</sup> LINE-1 RNA was detectable in teratocarcinoma cells but not in HeLa or other human cell types. The teratocarcinoma cell RNA was shown to be a mixture of transcripts from a large number of human LINE-1 elements. *In vitro* experiments have shown that at least several of these RNAs can be messenger RNAs, yielding relatively large amounts of the product of the first open reading frame. Preliminary data suggest that the relatively low level of translation of the second open reading frame depends on reinitiation of translation rather than suppression of the stop codons that separate the two frames.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00333-25 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Biochemical Basis for Defective Differentiation in Granulocytic Leukemia

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

W. H. Evans	Research Chemist	LB	NCI
S. M. Wilson	Biologist	LB	NCI

## COOPERATING UNITS (if any)

J. Bednarek, Hematology, Oncology Section, Walter Reed Army Medical Center  
 M. Mage, LB, DCBD, NCI

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Cellular Regulation Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1

## OTHER:

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.)

The main thrust of this work is to develop biochemical methods for the early diagnosis of granulocytic leukemia and methods for inducing leukemic cells to develop some or all of their functional properties as a means of partially or completely restoring host defense mechanisms in leukemia patients. Work is first aimed at establishing which of the many biochemical steps involved in normal granulocyte differentiation are controlled by humoral regulators. The results will be compared with those obtained from similar studies on leukemic cells at corresponding stages of maturity in order to determine the nature and potential reversibility of the arrested differentiation steps. Biochemical analyses are carried out on mature and immature granulocytes isolated from blood and bone marrow and the effects of cell regulators on granulocyte differentiation, as measured by changes in the synthesis of specific cellular components, are studied in a defined culture system previously developed in this laboratory. Possible relationships between transforming genes in leukemic myeloblasts and factors involved in the regulation of normal granulocyte differentiation are under investigation.

Project DescriptionObjectives:

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

Major Findings:A. Factors in Human Serum Affecting Human Neutrophil Precursor Maturation

Studies in collaboration with Dr. Jana Bednarek and Dr. Robert Knight of the Walter Reed Army Hospital are aimed at defining the nature of the factors in human serum that regulate bone marrow granulocyte end-stage maturation. Previously, we showed that we could prepare partially purified human granulocyte maturation factor (GMF), free of granulocyte maturation inhibitory factor (GMIF), from normal human serum using DEAE-fractogel displacement chromatography. The GMF produced by this step has an artificially high molecular weight (>300,000) as determined by size exclusion HPLC (SE-HPLC). Our more recent findings indicate that when human serum is analyzed directly by SE-HPLC GMF emerges with the included protein fraction in the 80,000-90,000 molecular weight range. As a possible explanation for this molecular size difference it occurred to us that the carboxymethyl dextran displacer used for the DEAE-fractogel step might be binding to the proteins in the GMF fraction on the chromatographic column and then causing a complex to form when the GMF was eluted from the column with 0.5 M NaCl. To test this possibility we repeated the DEAE-fractogel step except this time we omitted the displacer. As before, a large peak of inactive protein emerged in the unbound fraction and the GMF activity could be step-eluted from the column with 0.5 M NaCl. Subsequent analysis by SE-HPLC of the GMF activity from this step showed that this activity was no longer excluded from the SE-HPLC column but behaved like the GMF activity in whole serum and emerged with the included proteins in the 80,000-90,000 molecular weight range.

Work is progressing on the development of a preparative scale procedure to purify GMF from human serum to homogeneity. Because of the scarcity of human bone marrow donors and the unreliability of adequate yields of marrow cells from this source, we are now using guinea pig bone marrow granulocyte precursors as target cells in our screening assays for human GMF activity in chromatographic fractions. We find that if we separate transferrin from the GMF-containing fraction of human serum in the first step of purification, we can use guinea pig cells in the assay since these cells give essentially the same maturation response to the human GMF activity as do human cells.

For the first step of our preparative-scale GMF purification procedure, we are using DEAE-fractogel chromatography at pH 7.0. Human serum (25 ml) is dialyzed against the column starting buffer (10 mM sodium phosphate, pH 7.0) and applied to the column followed by starting buffer until most of unbound protein is removed from the column. This solution is then followed by a linear sodium chloride gradient from 0 to 0.5 M NaCl in starting buffer. Most of the GMF activity emerges at the beginning of the gradient. The active fractions contain 18% of the starting protein and are well separated from the transferrin fraction which emerges with the unbound proteins. Analysis of the proteins in the active fractions by SDS-gel electrophoresis showed that these fractions consisted mostly of albumin and a variety of minor proteins.

For the second step of the purification procedure, the active fractions from step 1 are pooled and dialyzed against 0.05 M Tris-HCl buffer (pH 8.6) and applied to another DEAE-fractogel column equilibrated with the same buffer. The unbound proteins are washed through the column with starting buffer and the bound proteins are eluted from the column using a linear sodium chloride gradient of 0 to 0.2 M NaCl in starting buffer. Under these conditions, the GMF activity emerges in the early part of the gradient and is well separated from the main protein peak. SDS-gel electrophoresis of the bound protein fractions from this step showed that the GMF activity was free of albumin. Approximately 82% of the GMF activity in serum was recovered in this step and we estimate that the activity is purified about 250-fold. The pooled GMF fraction from this step contains 4.6 mg of protein or about 0.33% of the starting serum protein.

#### B. Purification of GMF from bovine calf serum

Our studies indicate that bovine calf serum also contains a granulocyte maturation factor that is active on human bone marrow granulocyte precursor cells. The dose response curve for this serum is similar to that obtained with normal human serum. We are planning to use bovine calf serum as a large scale source of GMF since it is highly likely that large amounts of serum will be needed in order to obtain adequate amounts of this protein for amino acid sequence studies and for developing a specific immunochemical assay for this new maturation factor. Toward this end we are now collaborating with Dr. Ron Torres of Hyclone Corporation. Our preliminary studies indicate that GMF can be obtained as a by product of a large scale purification procedure for bovine serum transferrin currently used by Dr. Torres. In this procedure bovine serum (2000 ml) is chromatographed on a CM-cellulose column at pH 6.0. Transferrin binds to the column and the GMF activity emerges with the unbound protein fraction which consists mostly of albumin and a variety of minor proteins. Attempts are now under way to further purify GMF in this fraction by DEAE-fractogel chromatography.

#### Patent Application

The following patent application was filed on Nov. 21, 1988: New Patent Application, "Human Neutrophilic Granulocyte End-Stage Maturation Factor and Its Preparation and Use." (NIH Case No. E-250-88; task order No. 25).

This patent application outlines the potential uses of GMF as a bio-regulatory pharmaceutical agent for treating human diseases involving defects in or suppression of granulocyte maturation such as in chronic and acute myeloid leukemias, and in stimulating granulocyte maturation in patients who have severe infections such as burn victims, or patients who receive bone marrow transplants to replace marrow cells destroyed by X-irradiation and/or chemotherapy.

Publications:

Evans WH, Wilson SM, Bednarek JM, Peterson EA, Knight RD, Mage MG, McHugh L. Evidence for a factor in normal human serum that induces human neutrophilic granulocyte end-stage maturation in vitro. Leukemia Res 1989; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00366-13 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Structure and Expression of Endogenous Retroviral Elements

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

E. L. Kuff	Chief, Biosynthesis Section	LB	NCI
K. K. Lueders	Chemist	LB	NCI
M. Falzon	Visiting Associate	LB	NCI
J. Fewell	Microbiologist	LB	NCI

## COOPERATING UNITS (if any)

E. Leiter, Jackson Laboratory, Bar Harbor, ME

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Biosynthesis Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

3.0

## OTHER:

1.0

## CHECK APPROPRIATE BOXES)

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

(a1) Minors      B

(a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

We are examining cellular mechanisms involved in regulation of intracisternal A-particle (IAP) expression in normal and transformed cells. As one aspect of the program, we are studying the interaction of trans-acting factors with cis-regulatory sequences in the IAP LTR. An apparently novel enhancer-binding protein fraction has been isolated by DNA affinity chromatography from nuclear extracts of transformed human and mouse cells. The fraction contains two binding components of 75 and 85 kDa, and has similar affinities for the so called "enhancer core" domains of SV40, polyoma virus, and an IAP LTR. Further structural and functional analysis of these proteins is continuing. We have also begun a study to determine the means by which particular elements of the multi-copy IAP gene family are selectively activated in different cell types. We have established that in normal cells such as thymocytes and B-cell lymphocytes, IAP expression is clearly under genetic control, since cells from mice of different inbred strains show characteristic variations in level and type of IAP gene products. When IAP expression in stimulated splenic B-cells of BALB/cPi mice is compared with expression in primary plasmacytomas induced in animals of this strain, it is evident that many new IAP elements have been recruited to the active gene pool in the tumor cells. We are interested in whether this phenomenon reflects a general compromise of cellular mechanisms responsible for selective gene activation.

## Project Description

### Objectives:

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

### Major Findings:

#### A. Trans-activating Factors for IAP Expression (Dr. M. Falzon)

IAP expression is regulated by interaction of cellular factors with sequence elements in the 5' LTR. The U3 region of mouse IAP LTRs contains at least five nuclear protein binding domains identified by DNase I and ExoIII foot-printing (see Annual Report 1988). Two of these domains have homology with the SV40 enhancer core; we refer to them as Enh1 and Enh2. Enh2 appears to be an important determinant of LTR activity, since in vitro methylation at a HhaI site in this binding region greatly reduces LTR promotion of a linked reporter gene (CAT) in transfected cells. Dr. Falzon has isolated from both human 293 and mouse MOPC315 cells nuclear proteins that interact preferentially with the Enh1 and Enh2 regions, as shown by ExoIII foot-printing. A 4000-fold purification with respect to the crude nuclear extract of 293 cells was achieved by sequential DNA affinity chromatography on heparin-sepharose and a multimerized oligonucleotide representing the Enh2 binding region from the LTR of the mouse genomic IAP element MIA14. The affinity-isolated fraction from each cell line contains two polypeptide species with apparent sizes of approximately 75 and 85 kDa. Glycerol gradient sedimentation and UV crosslinking both confirmed that binding activity is associated with components of this size. The proteins also interact in a sequence-specific manner with oligonucleotides representing the enhancer core sequences in SV40, polyoma virus and the MSV LTR. Quantitative binding studies show that the proteins bind to enhancer core domains from the MIA LTR, SV40 and polyoma virus with similar affinities (dissociation constants between 8 and  $16 \times 10^{-11}$  M/L). The protein(s) differ in size and stability from the 43 kDa heat-resistant enhancer core binding protein C/EBP isolated from liver by McKnight and co-workers, and may represent a novel type of transcription factor. Enh2 binding proteins are more abundant in 293 (human), MOPC315 (mouse) and COS7 (monkey) cells, all transformed with nuclear oncogenes, than in HeLa, 3T3, and CV1 cells. Current studies are directed towards (1) accumulation of sufficient amounts of binding protein(s) to permit micro sequencing and development of oligonucleotide probes for cDNA and/or gene cloning; and (2) development of a factor-responsive in vitro transcription system to study factor interactions and mechanisms of methylation inhibition.

#### B. Endogenous Retrovirus Expression in Diabetes

We have continued a collaboration with Dr. E. Leiter (Jackson Laboratory) on the possible role of endogenous retrovirus (IAP) expression in the pathogenesis of a genetically determined mouse diabetes (see previous Annual Reports of this project). During the past year, Dr. Leiter has established transgenic mice of the C57BL/6 inbred strain carrying a genomic IAP element (MIA14) under transcriptional control of an insulin gene promoter. The

construct, which was prepared in our laboratory by J. Mietz, contains corrections of 2 frame shift mutations in the polymerase coding region of the original MIA14 genomic clone. In vitro translation of RNA synthesized from the modified clone has confirmed that both the gag and pol reading frames are open. C57BL/6 mice, which are resistant to the diabetogenic effects of the db gene, do not express IAPs in their pancreatic islet cells. This is in contrast to the islet cells of db susceptible strains, such as C57BL/Ks, which contain numerous IAPs. The questions to be answered are (1) whether IAPs will be expressed in islet cells of the transgenic C57BL/6 mice and (2) whether expression is associated with the development of diabetes when these animals are made homozygous for the db gene. The experiments are in progress.

### C. Selective Activation of IAP Genes in Different Cell Types

We have begun a study to determine if and by what mechanism(s) particular elements of the 2000 copy IAP gene family are selectively activated in different cell types. In normal cells such as thymocytes and B-lymphocytes, IAP expression is clearly under genetic control, since cells from different inbred strains show characteristic variations in level and type of IAP gene products. Because IAP expression has no apparent functional consequences for the cell, we postulate that activation of particular IAP elements may reflect their location in regions of active chromatin. Thus, transcription of particular elements may report the activities of neighboring genes in IAP-producing cells. The great enhancement of IAP expression in many tumor cell types could reflect a general compromise of cellular mechanisms responsible for selective gene activation, mediated by widespread genomic demethylation and/or increases in the levels of trans-acting cell regulatory factors to which IAP LTRs respond. Previous work (see earlier Annual Reports) has established that demethylation of CpG sites in the U3 region of the IAP 5' LTR is a prerequisite for IAP expression. We have developed a two-dimensional DNA electrophoresis method for assessing the number and variety of IAP elements that are demethylated in their 5' LTR. Thymus and splenic B cells from the same animal yield identical patterns of demethylated IAP elements by this technique, but the patterns differ markedly in lymphocytes from mice of different inbred strains. Thus particular subsets of IAP elements appear to be demethylated (and therefore available for transcription) according to the genetic background of the animal. We have begun experiments to define the number and variety of IAP elements actually expressed in normal lymphocytes. For this purpose J. Mietz has prepared a cDNA library from poly(A)RNA of LPS stimulated B cells from spleens of BALB/cPi mice. First strand synthesis was primed with an oligonucleotide representing a highly conserved region in the R region of the IAP LTR; thus many of the cDNA clones are expected to include the U3 region of the 3' LTR and extend for varying distances into the body of the IAP element. Sequencing of the cDNAs over a common 200-300 bp segment (or a longer region if necessary) should permit identification and enumeration of the different IAP elements represented in the poly(a)RNA fraction, since IAP elements generally differ from one another by at least 3% at the nucleotide sequence level. The BALB/c mouse was chosen for study because it is particularly susceptible to plasmacytoma induction. Transplanted plasmacytomas are known to express abundant IAPs and to have extensively demethylated genomic IAP elements. We have recently shown by 2-D electrophoretic analysis that IAP 5' LTRs are already heavily demethylated in primary plasmacytomas of BALB/c

mice and that a number of independently arising tumors have essentially the same pattern of demethylated elements. Evidently, extensive recruitment of new IAP elements into the active gene pool is a relatively early event in progression of plasma cell tumors.

Publications:

Falzon M, Kuff E. Multiple protein binding sites in an intracisternal A particle long terminal repeat, J Virol 1988;62:4070-7.

Kuff EL. Factors affecting retrotransposition of intracisternal A-particle proviral elements. Banbury Report: Eukaryotic transposable elements as mutagenic agents 1988;30:79-89.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00945-16 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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
K. Takeda	Visiting Fellow	LB	NCI
E. Schalk	Guest Researcher	LB	NCI
V. Shah	Visiting Fellow	LB	NCI
K. Hadley	IRTA Fellow	LB	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Biological Interactions Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

6

## PROFESSIONAL:

5

## OTHER:

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 suggested that guinea pigs that are vitamin C deficient and those that have been fasted, but supplemented with vitamin C, are equivalent with respect to the mechanisms responsible for decreased collagen and cartilage proteoglycan synthesis. Our previous studies provided considerable evidence for this proposal, including almost identical changes in circulating hormones and growth factors in the two groups of animals. Sera from both groups also contain a circulating factor that inhibited DNA synthesis in 3T3 cells and collagen and proteoglycan synthesis in cultured primary chondrocytes. Since scurvy and fasting reduce the synthesis of several different types of collagen in various connective tissues, we examined the effects of sera from scorbutic and fasted guinea pigs on cultured adult human skin fibroblasts which synthesize type I collagen, as opposed to chondrocytes which synthesize type II. We found results similar to those in the chondrocyte system, demonstrating that IGF-I is involved in the regulation of at least two collagen types. We also have found that the presence of the inhibitor in fasted and scorbutic sera is correlated with an increase in low molecular weight IGF-I binding proteins that can inhibit binding of IGF-I to its receptor on 3T3 cells and human fibroblasts. We also are continuing our studies to elucidate the cause of the unusual collagen phenotype of 4-nitroquinoline-1-oxide transformed Syrian hamster embryo fibroblasts. These cells fail to synthesize the pro $\alpha$ (I) subunit of type I procollagen but continue to synthesize altered forms of the other subunit, pro $\alpha$ 2(I). Transfection of these cells with a cosmid containing the pro $\alpha$ (I) gene resulted in expression of pro $\alpha$ (I) mRNA, suggesting that there is a structural defect in a regulatory region of the endogenous gene rather than a problem with trans-acting regulatory factors. The pro $\alpha$ 2(I) chains synthesized by these cells appear to have a posttranslational modification in the collagenous domain of the procollagen chain. This modification occurs independently of the known posttranslational hydroxylations of proline and lysine.

## Project Description

### Objectives:

The objectives of this project are to elucidate the mechanisms regulating the expression of collagen and other extracellular matrix components and to define the role of insulin-like growth factors in this regulation.

### Major Findings:

#### A. Regulation of Collagen and Proteoglycan Synthesis During Scurvy and Fasting

Our previous results suggested that ascorbate-deficient and fasted, ascorbate-supplemented guinea pigs are equivalent with respect to the mechanisms by which collagen and cartilage proteoglycan synthesis are decreased. Furthermore, sera from these animals could transmit the defects in extracellular matrix synthesis to cultured chondrocytes, in the presence of ascorbate, and this effect resulted from the presence of an inhibitor. These sera also inhibited the stimulation of DNA synthesis in quiescent 3T3 cells by normal guinea pig serum. The inhibition of these processes in both cell types was reversed by IGF-I. Since the effects of ascorbate-deficiency and fasting on collagen synthesis are generalized, we examined the effects of scorbutic and fasted guinea pig sera on human skin fibroblasts which synthesize type I collagen, as opposed to chondrocytes which synthesize type II. We found a similar inhibition of collagen synthesis by these sera in the fibroblasts and inhibition was reversed by IGF-I, as in the chondrocytes. These results demonstrate that, in addition to the well established role of IGF-I in regulation of cartilage proteoglycans, the growth factor also regulates the synthesis of at least two different collagen types. They also provide further evidence that culture systems consisting of sera from scorbutic or fasted guinea pigs and connective tissue cells reflect the regulation of extracellular matrix synthesis observed in vivo and can be used to analyze the circulating factors involved in this regulation.

The ability of IGF-I to reverse inhibition of various cellular functions by fasted and scorbutic sera suggested that the inhibitor might be an IGF-I binding protein. This idea was substantiated by the finding that there was an increase in the binding of [<sup>125</sup>I]IGF-I to proteins in fasted and scorbutic sera. Furthermore, the increase was correlated with the inhibitory activity of the sera for DNA synthesis in 3T3 cells and collagen synthesis in chick embryo chondrocytes and human fibroblasts but not with their IGF-I content. Gel filtration of sera labeled with radioactive IGF-I revealed that the label was present in proteins of approximately 30-40 kDa. The results suggest that a low molecular weight IGF binding protein(s) different than the high molecular weight complex that carries most of the IGF in serum, is induced in scurvy and fasting. We currently are carrying out studies to determine whether the isolated binding proteins are responsible for the inhibition of IGF-I stimulated functions in cultured cells.

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

1. **Modified Pro $\alpha$ 2(I) Chains.** Our previous work showed that there were two polypeptides secreted by NQT-SHE cells that could be separated by ammonium sulfate fractionation and they were designated as N33 and N50 collagens. They differed from the pro $\alpha$ 2(I) chain in type I procollagen secreted by normal Syrian hamster fibroblasts in that they migrated more slowly during SDS/PAGE. The modification was retained in the collagenous domain after limited pepsin digestion of the procollagen forms. It also was present in a cyanogen bromide peptide (CB-3,5) that spans about 60% of the C-terminal end of the chain (60 kDa) and in two V8 protease-derived peptides of approximately 30 and 40 kDa, which also are derived from the C-terminal domain. Several lower molecular weight V8-derived peptides from these chains were identical to those from the normal chain, indicating that the modification was localized in the C-terminal domain. We recently determined that the modification is present only in the secreted N-collagens and not in the intracellular polypeptides, which suggests that it is a post-translational modification that occurs just prior to secretion. When proline and lysine hydroxylations were inhibited, the slower migration of the N-collagen chains, as well as their CB-3,5 peptides and the two V8 protease-derived peptides, persisted during SDS/PAGE. These results indicate that the modification does not involve proline or lysine hydroxylation and thus represents a new type of post-translational modification in the collagenous domain.
2. **Mechanism for Lack of Expression of the Pro $\alpha$ 1(I) Subunit.** Our previous results showed that failure of NQT-SHE cells to synthesize the pro $\alpha$ 1(I) subunit of type I procollagen was correlated with a decreased level of its mRNA, as measured by dot-blot hybridization with a cDNA probe and in a cell-free translation system. Since nitroquinoline oxide is a mutagen, the reduced expression of the gene could have resulted from a mutation in a regulatory element of the gene itself or in the gene for a trans-acting regulatory molecule. We approached this question by transfecting NQT-SHE fibroblasts with a cosmid containing the entire human pro $\alpha$ 1(I) gene. The cells were cotransfected with pSV2neo in order to provide a selectable marker. Neomycin(G-418) resistant clones were isolated and DNA from these clones was assayed for the presence of the cosmid by dot-blot hybridization with a <sup>32</sup>P-labeled probe specific for the cosmid (cos). RNA was prepared from cos<sup>+</sup> clones and dot-blot hybridization assays with a pro $\alpha$ 1(I) cDNA probe showed that there was expression of pro $\alpha$ 1(I) mRNA. The level of expression, however, was much lower than in normal hamster fibroblasts. Transient transfection experiments with the cosmid alone have confirmed that expression of pro $\alpha$ 1(I) mRNA can be increased in NQT-SHE. These results suggest that there is a defect in the endogenous pro $\alpha$ 1(I) gene of NQT-SHE cells, perhaps resulting directly from mutagenesis. To substantiate this conclusion, we are currently determining whether the pro $\alpha$ 1(I) mRNA expressed in NQT-SHE fibroblasts after transfection with the cosmid is human or hamster.

Publications:

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05202-22 LB

## PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Isolation, Fractionation and Characterization of Native Nucleoproteins

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

O. Wesley McBride	Chief, Cellular Regulation Section	LB	NCI
A. Bale	Biotechnology Fellow	LB	NCI
S. Olson	Biotechnology Fellow	LB	NCI
M. Wang	Visiting Associate	LB	NCI
A. Mitchell	Chemist	LB	NCI

COOPERATING UNITS (If any). Hatfield, F. Gonzalez, M. Bustin, L.M. Staudt, NCI; J. Chan, L. Kohn, NIDDKD; R. Eldridge, NINCDS; A. Notkins, NIDR; E.E. Strehler, Zurich; J. Idle, London, D. Yang, Georgetown U., M. Young, NIDR, R. Adelstein, NHLBI, P. Elwood, C. Klee, A. Fornace, I. Kirsch, U. Siebenlist, NCI, K. Uyeda, U. Texas, U. Meyer, Basel, K. Ozato, NICHD, H. Ozer, N.J. School of Med.

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Cellular Regulation Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.15

## PROFESSIONAL:

3.0

## OTHER:

1.15

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

A panel of human-rodent somatic cell hybrids previously isolated and characterized in this laboratory has been used for the chromosomal localization of human genes by Southern analysis of hybrid cell DNAs in collaboration with investigators at NIH and elsewhere. About 20-25 genes have been mapped by this procedure during the past year. These have included lymphokines, neoplastic breakpoints, and genes involved in DNA repair, cell adhesion and regulation of cell proliferation, transcriptional regulation, and cellular regulatory pathways. The gene size, organization, and copy number at each locus was also determined. Related observations included the localization of some of these genes near chromosomal breakpoints in neoplasms and the variable amplification of some loci exhibiting variation among individuals. Several of these genes have been more precisely localized on chromosomes by in situ hybridization of metaphase chromosome spreads.

As C.E.P.H. collaborators, Southern blots of DNA restriction digests of DNAs from 40 large kindreds have been prepared and used for regional gene localization by genetic linkage analysis using probes which detect restriction fragment length polymorphisms. About 20 genes on 7 different human chromosomes have been mapped by this method during the past year. These results now allow the evaluation of the role of several of these candidate genes in specific hereditary diseases.

Project DescriptionObjectives:

1) Human chromosomal mapping of protooncogenes and genes involved in DNA synthesis, carcinogen metabolism, and regulation of cell proliferation and gene expression, and understanding role of these genes in human neoplasia, 2) mapping the genes for hereditary cancer predisposition syndromes, and 3) developing a map of the human genome and identifying specific genes or gene alterations involved in hereditary diseases.

Major Findings:

A large panel of human-rodent somatic cell hybrids isolated and characterized in this laboratory continues to be used for the chromosomal localization of human genes in collaboration with investigators at NIH and elsewhere. During the past year, these studies have involved the mapping of lymphokines, neoplastic breakpoints, and genes involved in DNA repair, cell adhesion and regulation of cell proliferation, transcriptional regulation, and cellular regulatory pathways. Probes used in these studies have also routinely been evaluated for detection of restriction fragment length polymorphisms (RFLPs) for subsequent use in mapping by genetic linkage analysis. Some of the genes mapped [and collaborators] during this interval have included: aspartyl tRNA synthetase [D. Yang, Georgetown U.]; proteoglycans I (biglycan) and II (decorin) and sialoproteins BSP I (osteopontin) and BSP II [M. Young, NIDR]; cytochrome P450IIF [F. Gonzalez, NCI]; calcium-ATPase isoforms 1 and 3 [E. Strehler & E. Carafoli, Zurich]; two heavy polypeptide non-muscle myosin genes [R. Adelstein, NHLBI]; MFBP, a folate binding protein [P. Elwood, NCI]; H-2RIIBP transcriptional regulator [K. Ozato, NICHD]; two N-acetyl transferase genes [Urs Meyers, Basel]; genes for three polypeptides of the Pyruvate Dehydrogenase Complex, E1 $\alpha$ , E1 $\beta$ , & E3 [B.J. Song, LMMS, ALC]; calcineurin A [C. Klee, NCI]; Fru-6,2 kinase:Fru-1,6 bisphosphatase [K. Uyeda, U. Texas]; nucleolin [M. Srivastava, NHLBI]; breakpoints in two T-cell leukemias [I. Kirsch, NCI]; two genes activated by DNA damaging agents [A. Fornace, NCI]; a gene involved in regulation of DNA synthesis [H. Ozer, NJ School of MED]; a new collagen or collagen-like gene [S. Kimura, NCI]; and two lymphokines [U. Siebenlist, NCI]. The gene size, organization, and copy number at each locus has also been determined.

Various types of additional information beyond chromosomal assignments have been obtained in these studies. Calmodulin appears to contain multiple members of multigene families. The calmodulin probe we have studied in collaboration with S. Detera-Wadleigh detects a multigene family dispersed on five different chromosomes and this cDNA probe does not detect other calmodulin multigene families isolated in two other laboratories. This results from maximal nucleotide sequence divergence between these sets of multigene families within the constraints of almost complete amino acid sequence conservation. The genes for two isoforms of human Ca ATPase have been assigned to human chromosomes 1 and 12, and localized to specific chromosomal regions by three different methods. The organization and copy number of these genes has been investigated by Southern analysis with subfragments of the cDNAs and by varying the stringency of hybridization. The two genes have regions of sufficient nucleotide similarity to be detected under reduced stringency but this similarity is less than is found

between each of the human genes and their rodent counterparts. A third locus was detected on the human X-chromosome with the isoform 3 probe only under reduced stringency, and an internal EcoRI restriction site has been lost from this gene. The genes for two lymphokines present at a single locus on chromosome 17 were subsequently found to be linked at the molecular level. There are also reduplications of these two genes at the locus with different copy numbers in different individuals, and this is under study at the molecular (U. Siebenlist) and chromosomal (this lab) level. The location of these two lymphokine genes near chromosomal breakpoints in two different classes of neoplasms is intriguing and under further investigation. A collagen, or collagen-like, cDNA sequence cloned by S. Kimura has a different chromosomal location than all previously reported collagen genes indicating that it represents a new class of collagen.

Genetic linkage mapping in kindreds with nucleic acid probes which detect RFLPs is being used in this lab to: 1) identify the loci for hereditary diseases, 2) obtain regional chromosomal localization of cDNAs and genes which have been isolated, 3) improve the reliability and resolution of the current human linkage map with additional loci and 4) establish a map of genes in the human genome. We are collaborators in the Centre d' Etude du Polymorphisme Humain (C.E.P.H.) human gene mapping project and we are using the DNAs from the 40 large CEPH pedigrees for much of this work. Southern blots have been prepared on nylon membranes after digestion of most of these 500 DNAs with 10 different restriction endonucleases and used to map about 20 genes during the past year. These genes include: HMG-17 (a human non-histone chromosomal protein), Gly 10 (a glycine tRNA), and CaATPase isoform 3 on chromosome 1; DNA polymerase beta on chr.8; CaATPase isoform 1 on chr.12; TCRA (T cell receptor, alpha polypeptide), RS-2 (a cluster of 4 tRNAs), 1858 and 1828 (lab designation for two different T-cell neoplastic breakpoints on chr.14q32.1), and CKBB (creatine kinase, brain form) on chr.14; CYP2A, CYP2B, and CYP2F (3 subfamilies of cytochromes P450) and TRSP (opal suppressor tRNA) on chr.19; HMG-14 (non-histone chromosomal protein), SOD1 (superoxide dismutase 1, soluble), and GPX1P2 (a processed pseudogene of glutathione peroxidase 1) on chr.21; TSHR (putative functional TSH receptor), CYP2D (cytochrome P450, debrisoquine), and PDGFB (platelet derived growth factor beta polypeptide) on chr.22.

In situ hybridization of metaphase chromosome spreads is the third method used in this laboratory for human gene mapping. Regional localizations have been obtained recently for CaATPase isoform 1, CaATPase isoform 3, RS-2 (tRNA cluster), CYPOR (cytochrome P450 reductase), and N-acetyltransferase genes.

#### Publications:

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05203-21 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Immunochemical Purification and Characterization of Immunocytes and Components

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

M.G. Mage	Immunochemist	LB NCI
L.L. McHugh	Biologist	LB NCI
B. Nardelli	Visiting Fellow	LB NCI

## 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:

2.25

## PROFESSIONAL:

2.25

## 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.)

Our laboratory has a long standing interest in development of immunochemical methods for cell separation, for studying receptor-mediated activation of T cells, and in the role of T cells in tumor rejection. With respect to immune activation, we are particularly interested in developing methods for the direct measurement of the interaction of the T cell receptor with MHC antigens. As an outgrowth of these interests, we have recently been developing methods for constructing semisynthetic multivalent macromolecular reagents, for which we have coined the term "polygens". Polygens are high molecular weight soluble polymers conjugated to proteins such as antigens, antibodies, toxins, or lymphokines. We believe that polygens, by increasing the effective valence of protein epitopes, may help to measure low affinity molecular interactions such as that between the T cell receptor and MHC antigens, and that such studies may help us understand the molecular requirements for activation of T cells. During this year, we have improved our methods for making polygens, and have developed a general reagent: polyacrylamide-streptavidin (PASA) for use in making such multivalent conjugates. We have used PASA to make heterologating antibody conjugates, and to make conjugates that coat tumor cells with Class I MHC antigens. Our studies of the enhanced immunogenicity of "xenogenized" (mutagenized) cell lines have led to the identification of an altered form of the gp70 antigen on xenogenized cells.

Project DescriptionObjectives:

The goals of this project are to develop and improve methods for studying receptor-mediated cellular activation, for targeting tumor cells for attack by CTL, and to apply improved understanding of T cell activation, together with new methodologies, to the development of improved vaccines for diseases such as malaria and AIDS, and for better targeting reagents and immunotoxins for treating cancers.

Major Findings:A. Development of a General Method for Making Soluble Multivalent Macromolecular Conjugates

"PASA" (polyacrylamide-streptavidin) had been synthesized by attaching multiple molecules of streptavidin to single molecules of soluble linear polyacrylamide. PASA can in turn bind biotinylated single proteins or mixtures to create multivalent soluble high molecular weight homo- or heteroconjugates. Such conjugates are potentially useful as vaccines, targeting reagents, immunotoxins, and amplification reagents for protein and DNA assays. We have applied for a patent for PASA.

In preparing PASA, we have used soluble linear polyacrylamide because it is available in bulk quantities, and can be modified to adjust the number of activated groups introduced for coupling to protein. This linear molecule is easier to characterize and maintain in soluble form than are branched molecules. We wanted to avoid intermolecular crosslinking, to make it easier for most if not all of the attached proteins to contact the cell surface. For this reason, we use a large excess of streptavidin in the coupling step.

The first step in preparing PASA is a mild alkaline hydrolysis of the polyacrylamide to convert a limited number of the amide groups to carboxyl residues. After dialysis to remove ammonia, we activated the carboxyl groups with a carbodiimide (CDI), in order to form peptide bonds with amino groups on the streptavidin. (Although CDI has been used for a long time to crosslink proteins in aqueous solution, an active intermediate is not isolated. Instead the proteins are mixed together with the CDI and as soon as an activated carboxyl group forms, it can form a peptide bond with an amino group.) We wished to avoid this situation, because we did not want to have intermolecular crosslinking. So we investigated to see if we could find aqueous conditions where the active intermediate was sufficiently stable to be separated from excess CDI prior to reaction with the streptavidin. By varying pH, time, and temperature, we found that the polyacrylamide with activated carboxyl groups could indeed be formed at pH 3 at 0 degrees, and separated from excess CDI by passage through a small Sephadex G25 column at 0 degrees. At this pH and temperature, the activated groups have a half life of about 1/2 hour.

The activated polyacrylamide is next mixed at 0 degrees with a large excess of streptavidin at pH 8.5. Coupling is complete in a few minutes. The conjugate of polyacrylamide to streptavidin (PASA) is separated from excess streptavidin by HPLC size exclusion chromatography. We are currently collaborating with several groups to test PASA as a backbone for making vaccines, immunosuppressive reagents, and amplification reagents for ELISA and for detecting biotinylated DNA probes.

B. Synthesis and Use of PASA-Based Heteroconjugates for Cellular Heterologation and for Coating Tumor Cells with MHC Class I Antigen Molecules

By reacting PASA with the appropriate biotinylated antigens and antibodies, we have prepared soluble multivalent heterologating antibody conjugates that can bind either of two antigenically distinct cell lines. For example, PASA was incubated with a mixture of biotinylated monoclonal antibodies to the H-2k and H-2d Class I MHC molecules. The resulting heteroconjugate was able to bind to either RDM4 cells (H-2k) or P815 cells (H-2d), although the antibodies singly could bind to only one cell type. By using a rat anti-H-2d and a mouse anti-H-2k, the presence of both antibodies in the cell-bound heteroconjugate could be demonstrated and distinguished by flow cytometry.

PASA has also been used to prepare reagents for labelling murine tumor cells with MHC antigens. Monoclonal antibodies to an antigen on the tumor cell surface and MHC Class I antigens are biotinylated and mixed. They are then coupled to PASA. In this fashion we have prepared heteroconjugates that can (for example) react with H-2k cells and coat them with H-2d antigen. The presence of the added H-2d antigen on the H-2k cells can be detected by flow cytometry. We are currently modifying PASA-based conjugates to have sterically unhindered Class I MHC antigens attached to them, the better to measure interaction with T cell receptor molecules and for tumor targeting.

C. Identification of a Cell Surface Antigen Specific for Xenogenized Tumor Cells

"Xenogenization" (i.e. mutagenization of tumor cells to increase their immunogenicity) is a potential approach to cancer immunotherapy of tumors that fail to elicit an effective immune response. In collaboration with the Institute of Pharmacology of the University of Perugia (supported by Italy-USA joint project CNR (Italy) contract number N.86.0031.04), and with Drs. E. Appella and S. Ullrich (LCB, DCBD, NCI) an apparent variant or mutant form of the gp70 protein has been identified in cell surface extracts of xenogenized cells by immunoprecipitation of labelled proteins, followed by 2D gel electrophoresis. We are currently working on the isolation of the mRNA for the putative mutant gp70 molecule, in order to prepare a DNA copy followed by a sequence, in order to understand the nature of this variant molecule. It is interesting to note that evidence has been found for gp70 molecules as targets for CTL in human hematological malignancies (Szabo et al., Acta Microbiol Hung, 1988;35:25-33).



Publications:

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Grohmann V, Romani L, Principato GB, Puccetti P, Mage MG, Fioretti MC. Generation of monoclonal antibodies to a chemically xenogenized murine lymphoma, *Pharmacol Res Commun* 1988;20:443-4.

Nardelli B, McHugh L, Mage MG. Polyacrylamide-Streptavidin: a novel reagent for simplified construction of soluble multivalent macromolecular conjugates, *J. Imm Methods*; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05214-18 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

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

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

S. H. Wilson	Medical Officer	LB	NCI
B. Zmudzka	Visiting Associate	LB	NCI
P. Kedar	Visiting Fellow	LB	NCI
S. Widen	IRTA Fellow	LB	NCI
J. Abbotts	IRTA Fellow	LB	NCI
P. Becerra	Guest Research	LB	NCI
E. Englander	Visiting Fellow	LB	NCI

## COOPERATING UNITS (if any)

A. Fornace, NCI; K. Williams, Yale; R. Karpel, Univ. of Maryland; A. Matsukage, Aichi Can. Gen., Nagoya, Japan; J. Siedlecki, Inst. Oncology, Warsaw, Poland

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Nucleic Acid Enzymology Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.9

## PROFESSIONAL:

3.5

## OTHER:

.4

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

We continued biochemical studies of mammalian DNA replication proteins. A cDNA for  $\beta$ -polymerase has been subcloned in expression vectors; the protein has been overproduced in *E. coli*, purified in mg quantities and sequenced. Structure-function aspects and *in vitro* DNA repair activities of the recombinant enzyme are being studied. Genomic DNA spanning the gene for human  $\beta$ -polymerase was further characterized. The promoter region was studied by detailed deletion and site mutagenesis using a transient expression system. In other work, we purified a transcription factor for the  $\beta$ -polymerase gene. *In vitro* transcription studies of the gene are under way. A tissue survey of the rat indicated tissue-specific expression of the mRNA with testes containing much more than other tissues. We found that the mRNA is induced in a rodent cell line following treatment with the DNA damaging agent MNNG.

## Project Description

### Objectives:

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

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

### Major Findings:

#### A. Structure and Function of Mammalian DNA Polymerases

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

##### 1. Human $\beta$ -Polymerase

- a. DNA polymerase  $\beta$  ( $\beta$ -pol) is a housekeeping enzyme considered to be involved in DNA repair in vertebrate cells. In many cell lines,  $\beta$ -pol mRNA levels are typical of other cases of low-abundance, housekeeping gene mRNAs. However, differential expression of  $\beta$ -pol is found in some cell lines and tissues. For example, survey experiments of rodent tissue reveal that liver contains slightly less  $\beta$ -pol mRNA than most other tissues, whereas testes contain much more than other tissues and perhaps 50-fold more than liver; some human cell lines, such as teratocarcinoma NTerad2 and the adenovirus E1A/E1B transformed line 293, have about a 5-fold higher level of the  $\beta$ -pol transcript than reference cell lines such as fibroblast AG1522. Further, induction of  $\beta$ -pol mRNA level (4-fold) is seen within four hours after treatment of CHO cells with the single-base alkylating agents MNNG, MMS, or AAAF; by contrast, induction is not seen with other DNA damaging agents.

To ascertain whether differential mRNA expression could be studied at the level of the  $\beta$ -pol promoter, fusion gene constructs were prepared with the human  $\beta$ -pol core promoter (-100bp) linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. It was found that  $\beta$ -pol promoter activity in transient expression experiments is much higher in MNNG damaged CHO cells than in untreated cells and, in addition, that the  $\beta$ -pol promoter can be strongly activated by transcriptional control proteins such as E1A/E1B; note that these proteins are constitutively expressed in the 293 cell line mentioned above.

Proteins in crude nuclear extract capable of binding to the human  $\beta$ -pol promoter were surveyed by DNase I footprinting with the core promoter as probe. The results indicate a similar footprinting pattern for nuclear extract from rodent tissues or from various cell lines representing differences in mRNA expression or in ability to support fusion gene expression. The predominant footprinting corresponds to a decanucleotide palindromic element at -49 to -40 in the  $\beta$ -pol promoter, plus several flanking residues on each side. From mutagenesis experiments, this decanucleotide palindromic element (GTGACGTAC) is known to be required for activity of the  $\beta$ -pol-CAT fusion gene.

In order to purify protein(s) binding specifically to the palindromic element, a gel mobility shift assay was employed. An oligonucleotide corresponding to the palindrome and flanking residues was used as probe. Palindrome binding protein was purified from a bovine testes nuclear extract in a yield of approximately 3 $\mu$ g protein/120g tissue. The purified protein stimulates *in vitro* transcription of the  $\beta$ -pol promoter and, as expected, binds tightly to the palindrome region of the core promoter. cDNA cloning and further biochemical studies of the purified palindrome binding protein are underway.

- b. Characterization of the domain structure of mammalian DNA polymerase beta was conducted. Large-scale overproduction of the rat protein in *Escherichia coli* was achieved, and the purified recombinant protein was verified by almost sequencing using tryptic peptides. This  $\beta$ -polymerase is both a single-stranded DNA (ssDNA) binding protein and a DNA polymerase consisting of one polypeptide chain of 334 amino acids. As revealed by controlled proteolysis experiments, the protein is organized in two relatively protease-resistant segments linked by a ~15-residue protease sensitive region. One of the protease-resistant segments represents the NH<sub>2</sub>-terminal 20% of the protein, and this NH<sub>2</sub>-terminal domain (of about 75 residues) has binding affinity for ssDNA-agarase equal to that of the intact protein. The other protease-resistant segment, representing the COOH terminal domain of ~250 residues, does not bind to ssDNA. Neither domain, tested as a purified protein, has substantial DNA polymerase activity. The results are consistent with the idea that the NH<sub>2</sub>-terminal domain is responsible for DNA binding activity of the intact protein and can interact with DNA in a "modular" fashion, not requiring the remainder of the protein for its binding activity.
- c. Pyridoxal 5' phosphate is a potent inhibitor of the DNA polymerase activity of recombinant rat DNA polymerase beta. Kinetic studies indicate that the mechanism of PLP inhibition is complex. In a lower range of PLP concentration, inhibition is competitive with respect to substrate dNTP, whereas at higher levels of PLP, several forms of enzyme combine with PLP and are involved in the overall inhibition; a possible model for these interactions during the catalytic process was suggested. Reduction of the PLP-treated enzyme with sodium [<sup>3</sup>H] borohydride results in covalent



incorporation of 4 mol of PLP/mol of enzyme, and the modified enzyme is not capable of DNA polymerase activity. The presence of dNTP during the modification reaction blocks incorporation of 1 mol of PLP/mol of enzyme, and the enzyme so modified is almost fully active. This protective effect is not observed in the absence of template-primer. Tryptic peptide mapping of the PLP modified enzyme reveals four major sites of modification. Of these four sites, only one is protected by dNTP from pyridoxylation. Sequence analysis of the tryptic peptide corresponding to the protected site reveals that it spans residues 68-80 in the amino acid sequence of the enzyme, with Lys 71 as the site of pyridoxylation. These results indicate that Lys 71 is at or near the binding pocket for the dNTP substrate.

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

#### Publications:

Widen S, Kedar P, Wilson SH. Human  $\beta$ -polymerase gene: structure of the 5' flanking region and active promoter, *J Biol Chem* 1988;263:16992-8.

Zmudzka BZ, Fornace A, Collins J, Wilson SH. DNA polymerase  $\beta$  mRNA: cell-cycle and growth response in cultured human cells, *Nucleic Acids Res* 1988;16:9587-96.

Abbotts J, SenGupta DN, Zmudzka B, Widen S, Wilson SH. Human DNA polymerase beta: expression in *E. coli* and characterization of the recombinant enzyme. In: Moses RE, Summers WC, eds. DNA replication and mutagenesis. American Society of Microbiology Press, 1988;55-67.

Wilson SH, Abbotts J, Widen S. Progress toward the molecular biology of DNA polymerase  $\beta$  (review), *Biochim Biophys Acta* 1988;949:149-57.

Fornace A, Zmudzka B, Hollander MC, Wilson SH. Induction of DNA polymerase  $\beta$  mRNA by DNA damaging agents in chinese hamster and ovary cells, *Mol Cell Biol* 1989;9:851-3.

Basu A, Kedar P, Wilson SH, Modak MJ. Active site modification of mammalian DNA polymerase  $\beta$  with pyridoxal 5' phosphate: mechanism of inhibition and identification of a deoxynucleoside 5'-triphosphate binding site residue, *Biochemistry*; in press.

McBride OW, Kozak C, Wilson SH. Mapping of the gene for DNA polymerase  $\beta$  on mouse chromosome 8, *Cytogenet Cell Genet*; in press.

Nowak R, Kaczmarek L, Siedlecki JA, Zmudzka BZ, Wilson SH. Levels and size complexity of DNA polymerase  $\beta$  mRNA in rat regenerating liver and other organs, *Biochim Biophys Acta*; in press.

Wilson SH. Gene regulation and structure-function studies of mammalian  $\beta$ -polymerase (review). In: Strauss P, Wilson S, eds. The eukaryotic nucleus: molecular structure and macromolecular assemblies. Caldwell, NJ, Telford Press; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05231-15 LB

<b>PERIOD COVERED</b> October 1, 1988 to September 30, 1989		
<b>TITLE OF PROJECT</b> (80 characters or less. Title must fit on one line between the borders.) Role of Subunit Interactions in Enzyme Chemistry and Cellular Regulation		
<b>PRINCIPAL INVESTIGATOR</b> (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
C. B. Klee	Chief, Protein Biochemistry Section	LB NCI
M. H. Krinks	Chemist	LB NCI
D. Guerini	Visiting Fellow	LB NCI
Wei Xi Qin	Visiting Fellow	LB NCI
M. P. Strub	Visiting Fellow	LB NCI
J. Mackall	Guest Researcher	LB NCI
P. Stemmer	IRTA	LB NCI
J. Maune	Guest Researcher	LB NCI
R. Ochia	Guest Researcher	LB NCI
<b>COOPERATING UNITS</b> (if any) Dr. J. Shiloach, NIAMMD; Drs. A. Bax and M. Ikura, NIDDKD; Dr. K Beekingham, Rice Univ., Houston, TX; Drs. W. Hahn and J. Sikela, Colorado Health Sci. Center, Denver, CO; Dr. P. Cohen, University of Dundee, Dundee, Scotland.		
<b>LAB/BRANCH</b> Laboratory of Biochemistry, DCBD		
<b>SECTION</b> Protein Biochemistry		
<b>INSTITUTE AND LOCATION</b> National Cancer Institute, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b>	<b>PROFESSIONAL:</b>	<b>OTHER:</b>
7.5	7.0	0.5
<b>CHECK APPROPRIATE BOXES)</b>		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK</b> (Use standard unretarded type. Do not exceed the space provided.) The mechanism of stimulus-response coupling mediated by $Ca^{2+}$ and calmodulin was studied at two levels: the modulation of the $Ca^{2+}$ signal by calmodulin and the regulation of the activity of the calmodulin-stimulated protein phosphatase, calcineurin, by calmodulin. 1) Using calmodulin mutants synthesized in <i>E. coli</i> we obtained direct evidence for the cooperative binding of $Ca^{2+}$ to the two halves of calmodulin. Binding of $Ca^{2+}$ to the high affinity sites in the carboxyl terminal half of calmodulin reduces the affinity of the low affinity sites in the amino terminal half of the molecule and conversely binding to the low affinity sites alters the conformation of the carboxyl terminal half of the molecule. 2) The same mutants were used to demonstrate that $Ca^{2+}$ binding to the four $Ca^{2+}$ sites of calmodulin plays a role in the binding of calmodulin to calcineurin and its subsequent activation. Two different human cDNA clones of the large subunit of calcineurin have been isolated and sequenced. The deduced amino acid sequences of the proteins have allowed the characterization of the calmodulin-binding domain, revealed the extended similarity of the catalytic domain with protein phosphatases 1 and 2A and identified a novel structural element whose integrity is needed to maintain enzymatic activity.		

## Project Description

### Objectives:

To study the functional roles of protein subunits and protein-protein interactions. The system under investigation is the  $\text{Ca}^{2+}$ -dependent regulation of enzymes mediated by calmodulin. Emphasis is on the mechanism of the regulation of the  $\text{Ca}^{2+}$ -dependent stimulation of the protein phosphatase, calcineurin, by calmodulin. These studies are undertaken to elucidate the roles of the two second messengers,  $\text{Ca}^{2+}$  and cAMP, in the regulation of cell function.

### Major Findings:

#### Calmodulin and Calcium Regulation of Cellular Activity

##### A. Interaction of Calmodulin with Calcium

These studies were undertaken to obtain insight into the molecular mechanism of the  $\text{Ca}^{2+}$ -dependent activation of many different enzymes by calmodulin. Previous studies carried out with D. Newton and J. Haiech showed that  $\text{Ca}^{2+}$  binding to calmodulin involves a positive cooperativity between two adjacent sites in the carboxyl and amino-halves of calmodulin and negative cooperativity between the two halves of calmodulin.  $\text{Ca}^{2+}$  binding to calmodulin is modulated by physiological concentrations of  $\text{Mg}^{2+}$  which enhances the negative cooperativity and thereby favors a stepwise conformational change of calmodulin. Using susceptibility to proteolysis as a probe for conformational changes, Julia Mackall has shown that at physiological concentrations,  $\text{Mg}^{2+}$  induces an alteration of the structure of the central helix of calmodulin. In collaboration with Kathleen Beckingham we have confirmed and extended these conclusions. A single mutation at Glu 104 or Glu 140 which results in a large decrease in affinity of sites III and IV for  $\text{Ca}^{2+}$ , increases the affinity of sites I and II. Thus, in normal calmodulin,  $\text{Ca}^{2+}$  binding to sites III and IV must induce an allosteric transition responsible for the negative cooperativity observed earlier. Conversely,  $\text{Ca}^{2+}$  binding to sites I and II of the calmodulin mutated at Glu 104 modifies the environment of Tyr 138 indicating that  $\text{Ca}^{2+}$  binding to the amino terminal half of calmodulin induces a conformational change in the carboxyl-terminal half of the molecule. We have therefore demonstrated that the two halves of calmodulin are not independent but respond to  $\text{Ca}^{2+}$  binding in a concerted manner.

##### B. Interaction of Calmodulin with Target Proteins

Our studies have been focused on the regulation of the  $\text{Ca}^{2+}$ /calmodulin-stimulated protein phosphatase, calcineurin. The regulation of this enzyme by  $\text{Ca}^{2+}$  is mediated by two different  $\text{Ca}^{2+}$ -binding proteins: an integral subunit of the enzyme, calcineurin B and calmodulin. In the absence of calmodulin, a small activation at low  $\text{Ca}^{2+}$  concentration ( $10^{-7}$  M) is mediated by calcineurin B. Using the calmodulin mutants described above, John Maune has shown that the  $\text{Ca}^{2+}$ -dependent stimulation of calcineurin by calmodulin depends upon  $\text{Ca}^{2+}$ -binding to calmodulin and not to calcineurin B.



The large stimulation produced by calmodulin is strongly cooperative (Hill coefficient=3-4) but requires more  $\text{Ca}^{2+}$  ( $K_{\text{act}} > 10^{-6}$ ). Mutants in site I that have preserved two high affinity sites require slightly higher  $\text{Ca}^{2+}$  concentration than does wild type calmodulin. The same substitution in site III results in loss of the high affinity sites and, consequently, a requirement for 10-50-fold more  $\text{Ca}^{2+}$  for activation. Mutation at either one of the  $\text{Ca}^{2+}$  sites results in decreased cooperativity (Hill coefficient  $< 2$ ). Thus, binding to both of the two  $\text{Ca}^{2+}$  binding units of calmodulin plays a role in the binding of calmodulin to calcineurin and its subsequent activation.

The characterization of the calmodulin-binding and regulatory domains of calcineurin A previously identified by M. Hubbard and M. Krinks was made possible by the determination of the primary structure of the protein. Danilo Guerini has cloned two calcineurin A cDNAs from human basal ganglia and brain stem mRNA. The complete sequences of the two calcineurin clones are identical except for a 54bp insert in the type I clone and different 3' ends including part of the coding sequence for the COOH termini of the two proteins. These findings suggest that calcineurin A consists of at least two isozymes which may result from alternative splicing events. The two enzymes differ at the carboxyl terminus which contains an inhibitory domain rapidly severed by limited proteolysis. With the exception of an 18 amino acid insert, the central parts of the molecule, which harbor the catalytic domains, are identical and show extended similarities with the catalytic subunits of protein phosphatases 1 and 2A, thus defining a distinct family of protein phosphatases. The 40 residue amino-terminal fragment, specific for calcineurin, contains a unique amino acid sequence which is also found in bovine brain calcineurin by peptide sequencing. A role in the calmodulin activation of calcineurin is proposed for this novel structural domain.

The two subunits of calcineurin and appropriately expressed mutants are being expressed in *E. coli* to elucidate the role of calcineurin B and calmodulin in the  $\text{Ca}^{2+}$  regulation of the enzyme while Marie Paule Strub is identifying sulfhydryl residues involved in the catalytic activity.

#### C. Physiological Roles of Calcineurin

A collaborative project with Dr. J. Thorner has been established to clone calcineurin in yeast and to study its role in the vegetative cycle of this organism. Enzymes, clones and antibodies prepared in the laboratory are made available to many investigators trying to study the role of protein dephosphorylation in many systems. Using some of our reagents, Dr. Catterall and his colleagues have recently obtained evidence for the specific dephosphorylation of the  $\text{Na}^+$  channel by calcineurin.

#### Publications:

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- Persechini A, Blumenthal DK, Jarrett HW, Klee CB, Hardy DO, Kretsinger RH. The effects of deletions in the central helix of calmodulin on enzyme activation and peptide binding, *J Biol Chem* 1989;264:8052-8.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05244-12 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Organization of Repeated DNA Sequences in Primates

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

M.F. Singer	Scientist Emeritus	LB	NCI
R.E. Thayer	Chemist	LB	NCI
T. Fanning	Expert; Special Volunteer	LB	NCI
V. Krek	Guest Researcher	LB	NCI
G. Swergold	Medical Staff Fellow	LB	NCI

## COOPERATING UNITS (if any)

Hector Seuanez, NCI-Frederick; Bill Modi, NCI-Frederick; Dr. Alan Scott, Johns Hopkins University Medical School

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Nucleic Acid Enzymology Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

4.75

## OTHER:

0.25

## CHECK APPROPRIATE BOX(ES)

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

B

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

The LINE-1 family of interspersed repeated DNA sequences that is ubiquitous in placental mammals and marsupials is very likely to be an active transposable element. Although LINE-1 segments lack terminal repeats of any kind, several observations indicated transposability: 1) the presence of target site duplications; 2) homology in one, highly conserved LINE-1 ORF to reverse transcriptase; 3) alleles that differ by the presence or absence of a LINE-1; 4) new LINE-1 insertions correlated with human mutations and tumors. Our experiments are designed to elucidate the mechanism of transposition of human LINE-1 elements. A central focus is the control of LINE-1 transcription. Experiments this year show clearly that transcriptional regulatory signals occur within the 5' end of the element. At least one important motif is in the first 100 bp from the 5' end; deletion of this segment from constructs containing the 5' leader of LINE-1 is accompanied by loss of expression of a fused reporter gene upon transfection. The level of expression of the reporter gene, using the intact 5' leader region, is almost 20 fold higher in human teratocarcinoma cells than in monkey kidney cells.

## Project Description

### Objectives:

Our aim is to understand the mechanism of LINE-1 transposition in the human genome, and to estimate the transposition rate in different cell types and at different stages of development. The working model for transposition mechanism is based on that of retroviruses and class I retrotransposons: 1) formation of full length-polyadenylated LINE-1 transcripts, 2) reverse transcription catalyzed by LINE-1 encoded enzyme, 3) insertion of the cDNA into a staggered break in genomic DNA, and 4) repair of the gaps at the junction between the cDNA and the genomic insertion site producing a target site duplication. However, the detailed mechanism of LINE-1 transposition must differ substantially from the mechanisms used by these well-studied elements. This is because LINE-1, as well as other class II retrotransposons, has structural properties distinct from the class I elements; in particular, the absence of LTRs.

Our current experiments approach these aims through analysis of the transcription and translation of human LINE-1 elements. In the current year, significant progress was made in the analysis of transcription, as described below. In addition, we have begun to investigate patterns of methylation of LINE-1 in different cell types, with the aim of understanding whether LINE-1 transcription is regulated by methylation. Finally, LINE-1 sequences continue to be used as probes for studies in evolutionary biology.

### Major Findings:

#### A. LINE-1 Family Transcription

Earlier we observed full-length, cytoplasmic, polyadenylated LINE-1 transcripts in human teratocarcinoma cells. These were characterized by primer extension and by analysis of cloned cDNAs. The 5' end of the bulk of the RNA coincides with the 5' terminus of full length genomic elements. The cDNA analysis indicated that many genomic LINE-1 elements are transcribed: no two of 19 cDNA clones analyzed had the same structure. These data raised questions about the nature of the promoter and other transcriptional regulatory elements that govern LINE-1 transcription, assuming that transcription is by RNA polymerase II. (The identity of the polymerase is likely, but strictly unproven for technical reasons.) Thus, a likely explanation was that the promoter and regulatory elements are all internal to the element and that many LINE-1 elements in human DNA have functional promoters, at least in teratocarcinoma cells.

We are investigating these questions by replacing the LINE-1 open reading frames (ORFs) with a convenient reporter gene, using recombinant DNA techniques. The 5' and 3' untranslated regions of the constructs are derived from the earlier cDNA clones because their analysis demonstrated that the LINE-1 RNA has a different consensus sequence than do randomly selected genomic clones. Initial experiments demonstrated that the approximately 900 bp long 5' leader region of one cDNA promotes expression of the bacterial chloramphenicol transacetylase (CAT) gene in human teratocarcinoma cells (line NTera2D1). However, the initial CAT constructs



were not ideal because the CAT mRNA is quite unstable in the cells and the 5' LINE-1 leader region was incomplete because none of the cDNA clones actually extended to residue 1 of the full length element.

During this year, a full length reporter vector was constructed by grafting the genomic consensus synthetic oligonucleotide for the first 11 base pairs onto the 5' end of the LINE-1 cDNA, cD16. cD16 itself begins at base pair 12. Then, the 5' end of the fully sequenced cD11, starting at base pair 152, was exchanged for the homologous region of cD16 (pLC1). The *E. coli* lacZ gene was fused in-frame to the beginning of the LINE-1 5' ORF (ORF1) (pLC1Z). All the LINE-1 ORFs were eliminated, but the final construct included a LINE-1 3' trailer region, with its polyadenylation signal. The 3' trailer element was derived from a recent LINE-1 insert into a human Factor VIII gene (kindly provided by H. Kazazian, Johns Hopkins University). This 3' trailer was chosen because it was complete, because it matched the sequence of a subset of LINE-1 cDNAs (subset Ta) we had previously suggested may define "active" LINE-1 elements, and because its recent transposition in the human genome also suggested it was derived from an active element (thereby confirming our earlier proposal). The complete recombinant vector, pLC1ZA, is being used for transfection into tissue culture cells and analysis of the products of LINE-1 transcription. A derivative of pLC1ZA, pLC1ZAD1, which lacks the entire 5' leader region (790 base pairs) is used as a control as is a construction in which the SV40 early promoter drives expression of the lacZ gene.

Activity of the lacZ constructs is being measured by assaying for beta-galactosidase activity in tissue cells transiently transfected by calcium phosphate co-precipitation. Two assays are being used: histochemical staining with a beta-galactosidase substrate that yields a colored product and spectrophotometric assay of the enzyme in cell extracts. No activity is detected with the pLC1ZAD1 control. pLC1ZA gives low, but reproducible levels of activity with mouse cells (NIH3T3, F9, and L); only a very low percent of the cells produce detectable beta-galactosidase. In CV-1 monkey cells, the LINE-1 5' leader permits expression of beta-galactosidase at about 5 percent of the level seen with the SV40 promoter. However, in human NTera2D1 cells, the LINE-1 5' end gives about 50 percent more activity than the SV40 promoter. The activity with the SV40 promoter is about the same in CV-1 and NTera2D1 cells. We do not know if the differential activity of the LINE-1 sequences in these cell lines is the result of species or cell type differences. Preliminary experiments are consistent with the initiation of LINE-1 controlled transcription at residue 1 of the 5' leader, as was found for the NTera2D1 cytoplasmic RNA. Moreover, deletion of the 5' most 100 base pairs of the 5' leader led to a loss of gene expression while the insertion of a 132 base pair DNA segment at residue 750 (as occurs in about 50 percent of human genomic LINE-1s) does not influence activity.

These studies indicate that the transcribable LINE-1 elements contain a promoter within the 5' leader. Unlike well characterized PolIII promoters, the important sequences are downstream of the transcription initiation site. At least some of the critical DNA regulatory motifs are within the first 100 base pairs of the LINE-1 element.

### B. LINE-1 Methylation

Experiments have focused on methylation in the GC-rich 5' leader region of LINE-1 segments. Southern blot analysis was carried out using the methyl-sensitive endonuclease HpaII and its methyl-insensitive isoschizomer MspI and probes representing the 5' terminal 650 bp or 5' terminal 200 bp of LINE-1. LINE-1 sequences in NTERA2D1 DNA were heavily methylated compared to DNA in several other tissue culture lines and primary lymphocyte DNA. However, a 300 bp HpaII fragment that annealed with both the 650 and the 200 probes was produced by NTERA2D1 DNA. This represented only a small portion of the total amount of the 300 bp fragment in the genome, as indicated by the MspI digests. Cell line JEG3 DNA, while less heavily methylated than NTERA2D1 overall, also showed such a band. It is possible that this hypomethylated set of LINE-1s is related to the detection of full-length, cytoplasmic, polyadenylated LINE-1 transcripts in NTERA2D1 cells and, at a lower abundance, in JEG3 cells. Thus, DNA from other cell lines, which do not contain detectable full length polyadenylated cytoplasmic transcripts, does not produce the 300 bp HpaII band.

### C. Comparative Study of Line-1

LINE-1 elements are being studied in neotropical primates of the infraorder Platyrrhini. Three families containing a total of 16 genera and 47 species are thought to have arrived in Central and South America about 45 million years ago. Probes derived from some of these animals are being used to study evolutionary history and construct molecular taxonomies and for analyses pertaining to the conservation genetics of endangered species. The work is also defining the highly conserved portions of LINE-1 elements as well as the variable regions, including the 5' end.

#### Publications:

Singer M, Skowronski J, Fanning TG, Mongkolsuk S. The functional potential of the human LINE-1 family of interspersed repeats. In: Lambert ME, McDonald JF, and Weinstein IB, eds. Eukaryotic Transposable Elements as Mutagenic Agents. New York: Cold Spring Harbor Laboratory Banbury Center, 1988;71-8.

Mongkolsuk S. Novel eukaryotic expression vectors which permit single-stranded replication in Escherichia coli and in vitro translational analysis of cloned genes, Gene 1988;70:313-9.

Singer MF. LINE-1 sequences: human transposable elements. In: Grunberg-Manago, ed. Evolutionary Tinkering in Gene Expression. New York: Plenum Press, 1988; in press.

Fanning TG, Modi WS, Wayne RK, O'Brien SJ. Evolution of heterochromatin-associated satellite DNA loci in the felids and canids (Carnivora), Cytogenet Cell Genet 1989; in press.

Modi WS, Fanning TG, Wayne RK, O'Brien SJ. Chromosomal localization of satellite DNA sequences among 22 species of felids and canids (Carnivora), Cytogenet Cell Genet 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05258-10 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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)

E.M. Paterson	Research Chemist	LB	NCI
J. Eldridge	Biochemist	LB	NCI
J. Rodriguez	NRC Associate	LB	NCI
B. Winter	Visiting Fellow	LB	NCI
L. DePonti-Zilli	Visiting Fellow	LB	NCI
Z.-Y. Lin	Visiting Fellow	LB	NCI
C. Dechesne	Visiting Fellow	LB	NCI
S. Nakamura	Visiting Scientist	LB	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Biochemistry of Gene Expression Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

8.50

## PROFESSIONAL:

8

## OTHER:

0.50

## CHECK APPROPRIATE BOX(ES)

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

B

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

We have isolated and characterized a number of muscle-specific and house-keeping genes that are differentially expressed during myogenesis. These include the alpha cardiac, alpha skeletal and beta cytoplasmic actin genes, and the myosin light chain 1f/3f gene. All except the beta cytoplasmic actin gene are expressed only in differentiated muscle. The beta cytoplasmic actin gene is suppressed in muscle. The cis acting sequence elements responsible for the regulated expression in these genes have been essentially characterized and we are currently in the process of identifying proteins that interact with these regulatory regions. Nuclear factor binding domains have been defined for the beta cytoplasmic actin promoter, the alpha cardiac actin promoter and on the light chain 1f/3f promoter. We are in the process of defining the various transcription factors and their binding domains on these muscle promoters. At present we have no evidence for any muscle specific factors, rather the factors we have identified via their binding domains are present in all cells in varying concentration. Regulation of the beta cytoplasmic actin gene is achieved through a sequence element in the 3' end of the gene which appears to interact with muscle-specific nuclear proteins to suppress transcription. We are trying to characterize this interaction more precisely. Recently we have isolated a gene that is involved in regulating the muscle phenotype in avian muscle, called CMD1, that appears to be the homologue of MyoD, the muscle determination factor from mouse. Studies on the characterization of the protein and its mechanism of action are in progress.

## Project Description

### Objectives:

We would like to understand the mechanisms involved in the control of eukaryotic gene expression during development and tissue formation.

### Major Findings:

#### A. Regulation of Actin Isoform Expression

Alpha cardiac actin is the major actin expressed first in differentiated embryonic skeletal muscle and it is eventually replaced by alpha skeletal actin in adult muscle. In contrast, beta cytoplasmic actin is expressed only in the dividing myoblast but is suppressed in differentiated muscle. Using a series of deletions and chimeric constructs we have been able to define regions in the alpha cardiac actin promoter and in the beta cytoplasmic actin gene involved in regulating actin expression during myogenesis. The alpha cardiac actin gene contains at least two distinct cis acting regions that appear to be involved in the negative regulation of the promoter as determined by in vivo transfection studies and in vitro transcription assays. Nuclear factors binding to the proximal region map over the TATA element between nucleotides -50 and -25. In the distal regions, factor binding spans nucleotides -136 to -112 over a region that includes a second CArG2 5' to the more familiar CCAAT box (CArG1). Nuclear factors binding to these different domains were found in both muscle and nonmuscle preparations, but were detectable at considerably lower levels in tissues expressing the alpha cardiac gene. In contrast, the concentration of the beta actin CCAAT box binding activity was similar in all extracts tested. The role of these factor-binding domains on the activity of the cardiac actin promoter in vivo and in vitro and the prevalence of the binding factors in nonmuscle extracts are consistent with the idea that these binding domains and their associated factors are involved in the tissue restricted expression of cardiac actin through positive and negative regulatory mechanisms. In the absence of negative regulatory factors these same binding domains act synergistically, via other factors to activate the cardiac actin promoter during myogenesis. Although the beta actin mRNA is down-regulated during myogenesis, the beta actin promoter confers constitutive expression when joined to heterologous genes transfected into a variety of different cell backgrounds, including differentiated muscle. Normal promoter activity is dependent upon the binding of a ubiquitous factor to the CCAAT-box element. Loss or reduction in factor binding correlates with a major reduction in promoter activity both in vivo and in vitro. The binding domain covers approximately 23 base pairs as determined by DNase footprinting. Methylation of A and G residues in and adjacent to the CCAAT box results in the loss of factor binding. Mutations across the binding domain indicate that the sequence GCCAATCAG within the domain is sufficient as a recognition sequence for factor sequence. Bandshift experiments demonstrate a predominant single band of similar mobility in nuclear extracts from various cells and tissues, with the exception of HeLa cells. The prevalence of the factor and its recognition sequence in a variety of promoters suggests that this factor has a common role in the transcriptional activation of several eukaryotic promoters.



Down regulation of the steady state level of beta actin mRNA is transcriptionally controlled, as determined by nuclear runon experiments, and this control is mediated by a 40 base pair sequence element just 5' to the canonical polyadenylation signal in the gene. Placement of this sequence in the corresponding position in both the alpha cardiac actin or neomycin-resistance gene in PSV2Neo or RSVNeo confers the beta actin mRNA regulatory pattern when these constructs are stably introduced into muscle cell lines such as C2C12 mouse myoblasts. This regulatory sequence is conserved in position in all the vertebrate beta actin genes sequenced and is not similar to any of the 3' processing adenylation for termination sequences described previously. The sequence element can be moved to any position in both orientations within the transcribed portion of the RSVNeo gene while maintaining the beta actin regulatory pattern for Neo expression in C2C12 cells. One position is 40 nucleotides beyond the polyadenylation signal yet the regulatory pattern is maintained. Muscle specific single-stranded DNA binding protein(s) appear to be involved in regulating beta actin gene expression.

#### B. Characterization of an Avian Myogenic factor

We have isolated the cDNA encoding a myogenic factor expressed in embryonic chick breast muscle by virtue of its weak hybridization to the mouse MyoD1 clone. Nucleotide sequence analysis and amino acid comparison defines this clone, called CMD1, as encoding a protein similar to mouse MyoD1. CMD1 encodes a polypeptide smaller than MyoD1, 298 versus 318 amino acids, respectively, and is 80% concordant by amino acid sequence overall. The basic and myc domains required for myogenic conversion of mouse 10T1/2 "fibroblasts" to myoblasts with MyoD1 are completely conserved in CMD1. CMD1 is just as efficient as the mouse homologue in myogenic conversion of 10T1/2 cells and coactivates the endogenous mouse MyoD1 gene in the process. The efficiency of myoblast conversion depends upon the levels of CMD1 expression and suggests that the cellular concentration of CMD1 plays a role in the onset of myogenesis. Transient expression of CMD1 in a variety of nonmuscle cells from different germ layer origins activates both cotransfected muscle specific promoters and, in some cases, endogenous muscle specific genes. BUdR treatment of chicken and mouse myoblasts reduces the expression of CMD1 and MyoD1, respectively, and may explain how this thymidine analogue inhibits myogenesis and the activity of transfected muscle specific promoters in BUdR treated myoblasts. Transient expression of CMD1 in BUdR treated myoblasts reactivates cotransfected muscle specific promoters. CMD1 activates muscle specific promoters in cotransfections regardless of cell type whereas "housekeeping" or constitutive promoters can be moderately activated, unaffected, or repressed depending upon the promoter and cell background. The rate and degree of myogenic conversion may be more restricted by cell phenotype than germ layer origin.

#### C. The Myosin Light Chain 1f/3f Gene

The myosin light chains (1 and 3) are encoded by a single gene containing two differentially used promoters producing mRNA transcripts containing unique and common exons. Transcription and splicing are regulated during differentiation. We have defined the minimal promoter elements for the

light chain 1 and light chain 3 transcripts by transfection studies with promoter deletions joined to the CAT reporter gene. The promoters are regulated in a tissue specific manner in primary cultures of embryonic chick breast muscle but are not properly regulated in rodent muscle cell lines. Each promoter element binds a common set and a unique set of nuclear proteins but none of the binding observed appears to be unique to muscle tissue. Although the mouse and human light chain genes appear to have specific enhancers in the 3' noncoding region of the gene, there is no evidence for a similar region in the chicken gene. The chicken gene was reported to have an enhancer in the 5' noncoding region but our studies have failed to confirm this result using the so-called enhancer. Methylation of particular C nucleotides block transcription from the promoters in CAT constructs and this block correlates with the loss of nuclear protein binding to particular regions in the promoter. Furthermore, *in vivo* methylation studies implicate the same methylated residues in promoter function. Further analysis of the promoters is under way.

#### D. Gene Regulation by Nerve Growth Factor

PC12 cells differentiate into neuronal cells in response to nerve growth factor (NGF). We have isolated the complete cDNA and gene encoding an mRNA that is induced 50-80 fold when PC12 cells are treated with NGF. The longest open reading frame in the cDNA encodes a polypeptide of approximately 72,000 daltons. LacZ fusion proteins with different regions of the cDNA open reading frame were used to produce polyclonal antibodies to study the protein induction process. The protein induced by NGF in PC12 cells is stored in secretory vesicles and released through the regulated secretion pathway. Furthermore, this protein or polypeptide, called VGF, is expressed in the hypothalamus of rats in the CNS. The mechanism of action of NGF on the promoter of the gene is under investigation.

#### E. Studies on the Yeast Myosin Gene

Using defined oligonucleotide probes to highly conserved regions in the myosin genes of UNC 54 of *C. elegans*, slime mold, *Acanthamoeba* and various vertebrate myosins, we have isolated the yeast homologue Myosin I gene. Gene replacement studies indicate that the gene is not essential for growth but plays a role in cytokinesis, nuclear migration, and cell wall deposition. Chitin plate formation is altered in the mutants implying that myosin, possibly via actin filaments, is involved in wall formation. The *Acanthamoeba* myosin gene cannot rescue the mutation and expression of this gene in wild type yeast results in the mutant phenotype. The head portion of the myosin molecule appears responsible for this phenotype since expression of the head alone behaves like the mutant. Expression of the rod portion of the *Acanthamoeba* gene has no effect. We are trying to isolate the myosin I yeast gene promoter but this has not proved successful.

#### Publications:

Quitschke WW, Eldridge JD, Paterson BM. Analysis of regulatory regions in the alpha cardiac actin promoter. In: Kedes L, Stockdale F, eds. Cellular and Molecular Biology of Muscle Development. New York, AR Liss Inc., 1989;677-89.

Quitschke WQ, Lin ZY, DePonti-Zilli L, Paterson BM. The beta actin promoter: high levels of transcription depend upon a CCAAT binding factor, *J Biol Chem* 1989;264:9539-46.

Quitschke WQ, DePonti-Zilli L, Lin ZY, Paterson BM. Identification of two nuclear factor binding domains in the chicken cardiac actin promoter: implications for the regulation of the gene, *Mol Cell Biol*; in press.

Lin ZY, Dechesne CA, Eldridge JE, Paterson BM. An avian muscle factor related to MyoD1 activates muscle specific promoters in nonmuscle cells of different germ layer origin and in BUdR treated myoblasts, *Genes and Develop*; in press.

Van den Pol AN, Decavel C, Levi A, Paterson B. Hypothalamic expression of a novel gene product, VGF; immunocytochemical analysis, *J Neuro Sci*; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05262-09 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Eukaryotic Gene Regulation and Function: The Metallothionein System

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

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

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Biochemistry of Gene Expression Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

8.75

## PROFESSIONAL:

8.5

## OTHER:

.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 mechanism by which copper induces yeast metallothionein gene transcription has been determined. In both the presence and absence of copper, the yeast cell constitutively synthesizes the ACE1 regulatory protein. The amino terminal domain of apo-ACE1 is in a randomly folded conformation that cannot bind to DNA and is therefore not functional. When the cell is confronted with a high concentration of copper, Cu(I) binds directly to ACE1 and causes a cooperative conformational switch into a form that can specifically recognize the metallothionein gene upstream regulatory sequences. Once ACE1 has bound to the DNA, the carboxy-terminal domain stimulates transcription by interacting with general transcription factors. The copper and DNA-binding domain of ACE1 is surprisingly similar to metallothionein itself, leading us to propose a novel "copper fist" model for its structure. In mammalian cells, a putative metallothionein gene regulatory factor has been purified and characterized, and the gene for an unusual zinc finger protein has been cloned.



## Projection Description

### Objectives:

We wish to understand how eukaryotic genes are activated or repressed in response to changes in the cellular environment and how the resulting gene products allow the cell to adapt to its altered conditions.

### Major Findings:

#### A. Yeast

1. Structure and Expression of ACE1, the Regulator of Yeast Metallothionein Gene Transcription. When yeast cells are confronted with a potentially toxic concentration of copper, they respond by increasing the transcription rate of the CUP1 locus which encodes a small, metallothionein-like copper binding protein. Several years ago, we devised a genetic strategy to isolate trans-acting regulators of metallothionein gene transcription. Our rationale was that mutant cells lacking a CUP1 activator protein would fail to make metallothionein and therefore be hypersensitive to copper toxicity (Thiele DJ, Hamer DH. In: Reznikoff WS, Burgess RR, Dahlberg JE, Gross CA, Record MT, Wickens M, eds. RNA Polymerase and the Regulation of Transcription. Amsterdam: Elsevier, 1987;431-5). Cloning and characterization of one such gene, called ACE1 (for Activation of CUP1 Expression), revealed it to be a trans-activator of CUP1 located on chromosome VII (Thiele DJ. Mol Cell Biol 1988;8:2745-52).

Fine mapping and DNA sequencing showed that ACE1 encodes a 25 kDa protein apparently consisting of two domains: an amino-terminal domain rich in basic amino acids and cysteines, and a carboxy-terminal domain with many acidic residues (Furst P, Hu S, Hackett R, Hamer DH. Cell 1988;55:705-17). To determine whether copper affects the synthesis of the ACE1 protein or its activity, measurements of ACE1 mRNA and an ACE1-lacZ fusion protein were performed. The results showed that ACE1 is constitutively expressed in the presence and absence of copper ions. Thus metal ions must activate ACE1 posttranslationally.

2. ACE1 is a Specific DNA-Binding Protein. To test whether ACE1 activates CUP1 indirectly, by a metabolic route, or directly by binding to the DNA, we synthesized the ACE1 protein in vitro and tested it for interaction with the CUP1 control sequences. Our results showed that ACE1 recognizes two upstream sequence elements of CUP1: UASc, which is a strong autonomous copper response element, and UASd, which is an auxiliary element. Experiments with point mutants showed that the same bases necessary for copper induction in vivo are required for DNA binding in vitro.

The binding of ACE1 to DNA is strictly dependent upon the addition of metal ions. Activation is specific for Cu(I) or the electronically analogous Ag(I) ion; all divalent ions tested failed to activate. Interestingly, silver was also found to activate CUP1 gene transcription in vivo. Thus there is a good parallel between metal activation in vivo and in vitro.

3. Metal Ions Alter the Conformation of ACE1. To determine how Cu(I) (and Ag(I)) activate the DNA-binding activity of ACE1, we used partial proteolysis to probe the conformation of the protein in the presence and absence of metal ions. Our results showed that apo-ACE1 exists in an apparently random conformation that is highly sensitive to proteases whereas Cu(I)-ACE1 is in a folded conformation that is partially resistant and produces discrete peptides. Thus the way that metal ions activate ACE1 is through a conformational switch.
4. Domain Structure. The domain structure of ACE1 was addressed by both genetic and biochemical experiments. We showed that a hybrid between the amino-terminal region of ACE1 and an activation domain of GAL4 could activate CUP1 transcription in the presence of copper. Conversely, a hybrid between the DNA-binding domain of ACE1 and the carboxy-terminal region of ACE1 activated GAL1 constitutively. Thus the amino-terminal region of ACE1 is a copper-dependent DNA-binding domain whereas the carboxy-terminal region is a copper-independent activation domain. This was confirmed in vitro using a truncated version of ACE1 containing only the amino-terminal domain. This 125 amino-acid peptide undergoes a conformational switch in the presence of Cu(I) and specifically binds to the CUP1 control sequences.
5. The "Copper Fist" Model. There are several intriguing structural similarities between the ACE1 regulatory protein and yeast copper-metallothionein itself: the presence of 12 cysteine residues, 10 of which are in CysXCys or CysXXCys pairs; the paucity of hydrophobic and aromatic residues; the specificity for the +1 oxidation state of copper; and the protease sensitivity of the apoprotein and resistance of the metalloprotein. Based on these similarities, we proposed a novel "copper fist" model in which the multiple cysteine residues chelate multiple copper ions to form a polynuclear Cu(I)-S core. We speculate that the folding of the protein around this metallothionein-like core forms DNA-binding "knuckles" rich in basic amino acids. We are currently testing this model by site-directed mutagenesis and biophysical studies. The use of a common structural motif for ligand and DNA binding has interesting implications for the evolution of homeostatic regulatory mechanisms.

## B. Mammalian Cells

1. Putative Metallothionein Gene Regulatory Factor. We have continued to work on the identification of mouse nuclear factors that bind to the metal response elements (MREs) of higher eukaryotic metallothionein genes. One such factor, called MBP-1, has been purified to the point of clear identification on an SDS gel and characterized in some detail. MBF-1 specifically binds to several MRE sequences including mouse MREc and trout MREa and MREb. Studies with point mutants show that the same bases are involved in metal activation in vivo and DNA binding in vitro. MBF-1 appears to be a metalloprotein in that it is inactivated by metal chelators. Preliminary experiments suggest that it can activate metallothionein gene transcription in vitro in a metal-dependent fashion. We are trying to clone the gene for MBF-1 by both antibody screening and peptide sequencing strategies.

2. Zinc Finger Protein. Many eukaryotic gene regulatory proteins have been shown to contain a zinc finger DNA-binding motif. Because of our interest in the role of metals in gene regulation, we have begun to characterize mouse proteins containing this motif. By screening a library at low stringency with a Kruppel zinc finger probe, we isolated several genes for putative zinc finger proteins and characterized one of these in detail. This clone is interesting in two respects. First, it is predicted to encode a protein which consists almost exclusively of the finger motif with only short flanking sequences. Second, it shows differential expression in transformed compared to normal cells. We are currently studying the intracellular localization, tissue distribution, metal dependence and possible functions of this protein.

Publications:

Furst P, Hu S, Hackett R, Hamer DH. Copper activates metallothionein gene transcription by altering the conformation of a specific DNA-binding protein, Cell 1988;55:705-17.

Kelly SL, Basu A, Teicher BA, Hacher MP, Hamer, DH, Lazo, JS. Overexpression of metallothionein confers resistance to anticancer drugs, Science 1988;241:1813-5.

Culotta VC, Hamer DH. Fine mapping of a mouse metallothionein gene metal response element, Mol Cell Biol 1989;9:1376-80.

Hamer DH. Essential and toxic heavy metal metabolism: the role of metallothionein. In: Trace Elements in Nutrition; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01.CB 05263-08 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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)

C. Wu	Visiting Scientist	LB	NCI
V. Zimarino	Visiting Associate	LB	NCI
J. L. Brown	Visiting Fellow	LB	NCI
S. Rabindran	IRTA Fellow	LB	NCI
G. Lavorgna	Visiting Fellow	LB	NCI
J. Clos	Guest Worker	LB	NCI
P. Becker	Guest Worker	LB	NCI
J. Westwood	Guest Worker	LB	NCI
B. Davis	Laboratory Worker	LB	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

8.25

## PROFESSIONAL:

7.75

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

## SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

This group has continued its study of sequence-specific DNA-binding proteins in *Drosophila*, with focus on the heat shock genes and the segmentation gene *fushi tarazu*, *ftz*. Molecular cloning of the heat shock gene transcriptional activator, or Heat Shock Factor, HSF, is close to completion. The HSF clone will be invaluable for further studies on the biochemical pathways involved in the cellular response to stress. New discoveries on the properties of inactive HSF show that activation of the pre-existing protein may occur *in vitro* by means of heat, low pH, or antibody induced conformational change of the protein. The nature of this conformational change could provide insights on the mechanism by which this one-component signal transducer can sense environmental stress and activate gene transcription. FtzF1, a DNA-binding factor that interacts with the *ftz* gene, has been purified and analyzed intensively. The function of Ftz F1 *in vivo* has been studied in transgenic flies, and these studies show that the factor is involved as a positive transcription factor in certain specific segments of the early embryo. Molecular cloning of the *ftzF1* gene is under way. The analysis of other factors involved in *ftz* gene transcription is being continued. The heat shock gene is being used as a model to analyze the cooperative or competitive interactions between nucleosomes and transcription factors by *in vitro* reconstitution experiments. These studies should clarify the role that nucleosome histone proteins have been suggested to play in controlling gene activity in higher cells.



## Project Description

### Objectives:

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

### Major Techniques Employed and Major Findings:

#### Regulation of the hsp70 Gene

##### A. Cloning the HSF Gene

We are cloning the HSF gene by partial microsequence analysis of HPLC purified peptides derived by trypsin cleavage of purified HSF. Using 5-10 micrograms of purified HSF, we have obtained good sequence information from 3 peptides, and prepared oligonucleotide probes deduced from the peptide sequences.

Screening of cDNA and genomic Drosophila libraries is under way.

##### B. Physical and Chemical Activation of HSF In Vitro

Cytoplasm from nonshocked Drosophila tissue culture cells can be activated in vitro to specific DNA binding by treatment with elevated temperature, acidic pH, and with the antibodies raised against HSF purified from heat shocked cells, i.e. anti-(active HSF) antibodies. These findings suggest that inactive HSF may be activated by a conformational change of the protein. Although it remains possible that the in vitro activation by heat or acidic pH is mediated through the activation of an enzyme activity that covalently modifies HSF in the crude cytoplasm, the fact that specific antibody to active HSF can also activate argues strongly that enzymatic modification is not an absolute requirement for activation. Nonetheless, it cannot be formally excluded at present that an enzymatic modification of HSF does occur as a result of heat and acidic pH activation. We are resolving the problem by purifying the inactive form of the protein for further studies of the activation process.

##### C. Purification of the hsp70 Gene TATA Box Binding Factor

In addition to the binding of the transcriptional activator HSF, one other transcription factor has been shown to bind in the nucleus to the heat shock promoter at the TATA sequence motif at position -30 upstream of the start of transcription. We had previously identified by exonuclease protection a TATA box binding activity in crude nuclear extracts of both shocked and unshocked Drosophila cells. We are purifying this activity (TATA factor) by means of conventional and affinity chromatography.

#### D. Chromatin Reconstitution of the hsp70 Gene

Based on previous chromatin mapping experiments, the TATA factor that is constitutively bound in vivo is proposed to play a critical role in the formation of a DNase I hypersensitive site, presumably by the exclusion of a nucleosome. Hence it will be of special interest to determine whether purified TATA factor can establish the same hypersensitivity in vitro by reconstitution in the presence of cloned heat shock gene DNA and histones. We are reconstituting the hsp70 gene in the presence and absence of prebound TATA factor with nucleosomal proteins supplied by chromatographically purified *Drosophila* histones and other assembly factors derived from *Xenopus* oocytes that are generally employed for this purpose. The integrity of the structural assembly will be gauged by nuclease digestion and nuclease protection assays. We will also determine the transcriptional ability of the assembled structures by introducing them as templates for in vitro transcription.

#### Regulation of the Fushi Tarazu Gene

##### A. Purification and Properties of ftzF1

Among the DNA binding factors that were identified by the systematic biochemical screen, one factor, FtzF1, whose binding site is TCAAGGTCGCCG, was chosen for further analysis initially because it displayed an interesting developmental regulation. The DNA binding activity is first observed in 1.5-4 hr embryos, when the ftz gene is expressed in the stripes. The early activity then decays, before reappearing again in an electrophoretically different form later in embryogenesis, at about 15 hr. The activity persists till hatching, and is present also in larval and adult life. FtzF1 has been purified to homogeneity from late embryos by conventional and affinity chromatography. This has been an arduous task that has necessitated continuous embryo harvest from the *Drosophila* mass colony for a year in order to obtain several kilograms of fly embryos as starting material. Repeated purification of ftzF1 is currently in progress in order to obtain ten micrograms of protein for peptide microsequence analysis. The sequences of the peptides will be used to design oligonucleotide probes for identification of the ftzF1 clone. The cloning of ftzF1 is also proceeding by screening of *Drosophila* expression libraries with a labelled binding sequence.

##### B. Significance of ftzF1 for ftz Gene Activity

Although the strong binding observed in vitro between ftzF1 and its binding site on the zebra element is suggestive of a functional interaction, it remained possible that the observed binding was an artifact of the in vitro assay. In order to determine the functional importance of the binding of ftzF1 to the ftz zebra element, we constructed 2 and 4 bp substitution mutations in the ftzF1 binding site. These mutations destroy the binding of ftzF1 to the DNA sequence on the zebra element. Most importantly, they also result in a decrease of ftz expression in stripes, especially in stripes 2, 3 and 6 of the embryo, when the mutant element is fused to the lacZ gene and expression assayed by X-gal staining in transgenic embryos (collaboration with Matthew Scott's laboratory, Boulder). The findings indicate that ftzF1 positively activates transcription of the ftz gene in stripes.

C. Analysis of Other ftz Gene Binding Factors

A second class of DNA-binding factors (ftzF2a, 2b, 2c, 2d) binds to a 68 bp sequence within the ftz zebra control element. The 68 bp region has the potential for multiple factor interactions. Biochemical fractionation of these factors is under way. The cloning of the ftzF2 group of factors is proceeding by the screening of expression libraries with the wild type 68 bp sequence and, as a control, a mutated 68 bp sequence that no longer interacts with the binding factors.

The analysis of transgenic *Drosophila* carrying mutations of the 68 bp sequence which impair the interactions with DNA-binding factors is being conducted in collaboration with the laboratory of Matthew Scott at Boulder, CO. This analysis will indicate the functional significance (activation or repression) of the ftzF2 group in the regulation of ftz expression in stripes.

Publications:

Wu C, Tsai C, Wilson S. DNA-affinity chromatography. In: Setlow J, ed. Genetic Engineering, vol 10. 1988;67-74.

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

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05264-08 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Characterization of a Mouse Repetitive Gene Family

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

K. K. Lueders	Research Chemist	LB NCI
E. L. Kuff	Chief, Biosynthesis Section	LB NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Biosynthesis Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOXES)

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

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Sequencing of IIB cDNAs and a genomic element has defined the structure of this subclass of IAP genes and revealed that they contain a long ORF potentially capable of encoding a 47 kDa polypeptide which includes the integrase gene. In vitro transcription/translation has shown that such a product is made. Proteins of 47 and 24 kDa have been detected by Western blotting in two myeloma cell lines with antiserum to a synthetic peptide from the integrase gene region. Presence of these proteins in cells appeared to correlate with IAP gene expression, but not with the level of type IIB element expression. Endonuclease activity and the 47 and 24 kDa immunoreactive proteins cofractionated in the same particulate fraction. The detergent solubilized endonuclease activity in MOPC 315 cells was concentrated in gradient fractions which included proteins in the 47 kDa size range. However, since activity could not be inhibited or complexed with the antibody and was present in cells not expressing IAP elements, a relationship between endonuclease activity and the 47 kDa protein has not yet been demonstrated.



## Project Description

### Objectives:

To study factors involved in control of expression of the endogenous proviral elements of the intracisternal A-particle (IAP) gene family and the effect of this expression on normal development and neoplastic transformation. Specifically, to determine whether type II IAP genes have a functional role in mouse cells and are expressed as protein products.

### Major Findings:

In some myelomas type IIB IAP elements (a subclass of IAP genes characterized by a specific 270 bp insertion, AIIins) have undergone marked amplification and transposition compared with embryonic cells and other myelomas. Type IIB RNA transcripts are the major IAP RNAs expressed in these myelomas. These elements have been shown to have extensive deletions in the gag and polymerase gene regions, but seem to have an intact integrase coding region. Since a similar degree of amplification of other classes of IAP elements has not been observed, we would like to understand how this particular subset is amplified and whether some property of the type IIB elements themselves has a role in this process. To answer this question we have prepared a cDNA library using RNA from MOPC 315 cells which have amplified type IIB IAP elements, characterized the expressed type IIB elements, and determined their coding capacity.

### A. Structure of Amplified IIB Elements

A MOPC 315 cDNA library was prepared from gradient fractionated poly(A)+ RNA enriched for 4 kb type IIB transcripts. Two cDNA clones, 106 and 111, were selected by reaction with the AIIins probe and cDNA 103 by reaction with a pol region probe. The clones were sequenced on both strands by the dideoxy method, using synthetic oligonucleotide primers. A type IIB genomic element isolated from BALB/c embryo DNA was also sequenced. These data have allowed us to elucidate the structure of the amplified elements.

The four clones were very similar to one another and differed substantially from type I clones. cDNA clones 106 and 111 differed by only 2 bp among 1069 with an overall homology of 99.8%. Both of these elements shared 99% homology with the independently isolated genomic IIB element but only 92.8 and 97.8% homology with the type I element in the regions 5' and 3' to AIIins, respectively. It is thus likely that one or multiple very closely related IIB elements are responsible for type II gene expression in MOPC 315 cells. Clone 103 did not include the region of AIIins, but clearly resembled a type IIB element (98.5%) more than a type I element (95.9%).

In the type IIB IAP elements, AIIins was inserted at position 1741 of the intact 7 kb IAP genome, and IAP sequences between that position and nucleotide 4391 were deleted. Position 1741 is located in the gag coding region of the IAP genome and position 4391 at the 3' end of the reverse transcriptase gene. There is a 66 bp duplication of sequence from the integrase gene immediately 3' to AIIins.

All the sequenced type IIB clones had 2 open reading frames (ORFs). Upstream of AIIins ORF 1 began in the same position as that in type I elements and continued for 325 bp. AIIins contained multiple stops in all frames. ORF 2 was 1340 bp long in cDNA 106 and the genomic IIB element. cDNAs 103 and 111 also had ORFs in the overlapping regions of 1131 and 730 bp, respectively. ORF 2 in type IIB cDNA 106 extended 27 codons beyond that found in the type IIB genomic clone and in the full-length genomic element MIA14. An identical extended ORF is present in an IAP element which transposed in the WEHI-3 T-cell leukemia cell line.

The protein coding capacity of the type IIB elements was confirmed by cloning the integrase regions from both cDNA 106 and the genomic element into a bacterial expression vector (pATH), in which the product was synthesized as an inducible fusion protein. Both constructs produced high levels of a 68 kDa protein detectable by staining of SDS-PAGE fractionated insoluble proteins from bacterial cultures. The size was consistent with translation of the IAP encoded integrase gene fused with the bacterial trpE gene.

#### B. Identification of Cellular Proteins Reacting with an Integrase Peptide Antibody

We had generated a polyvalent antiserum (EP20 Ab) by immunizing rabbits with an oligopeptide (EP20) predicted from the nucleotide sequence of the integrase gene in a full-length genomic IAP element. The oligonucleotide includes a region highly conserved among other retroviruses belonging to the same evolutionary lineage as the IAPs. Western blot analysis of the bacterial fusion products using EP20 Ab showed the products resulted from translation in the appropriate reading frame and that EP20 Ab could detect such proteins specifically.

With the specificity of EP20 Ab established, we tested for reactive proteins in MOPC 315 cells; proteins of 47 and 24 kDa were detected. The proteins were concentrated in a 100,000 x g particulate fraction, and did not cofractionate with IAPs. The reactions by Westerns were blocked when EP20 Ab was preincubated with EP20; while an unrelated peptide did not block the reaction, and EP20 did not block reaction of an unrelated antibody.

#### C. Endonuclease Activity

We had earlier detected a low level of very tightly bound endonuclease activity associated with gradient purified IAPs which we considered as an index of possible integrase gene function. Activity was measured by conversion of supercoiled (form I) plasmid DNA to relaxed and linear forms (forms II and III, respectively). The absence of proteins reacting with EP20 Ab in IAPs and their concentration in the 100,000 x g pellet led us to examine the latter fraction for endonuclease activity. This fraction had 20-fold more activity than the IAPs on a protein basis; the activity had identical properties to that associated with IAPs, except that it was partially solubilized by detergent treatment. Analysis of the solubilized material on a glycerol gradient showed that the peak of activity was associated with material 40-50 kDa in size. Although there appeared to be

correspondence between endonuclease activity and the presence of the 47 kDa protein detected with EP20 Ab, treatment of the fractions with EP20 Ab had no effect on this activity.

We compared the endonuclease activity associated with the 100,000 x g fractions isolated from two myelomas to determine whether the levels of activity vary in cells which express different subsets of IAP elements. If the type IIB elements were the source of integrase protein, MOPC 315 cells, which express primarily type IIB RNA, might be expected to have higher levels of endonuclease than a myeloma cell line such as MOPC 21, in which type I elements are the primary source of transcripts, although low levels of type II RNA are also detected. IAP fractions from the two cell lines had similar levels of endonuclease activity at several protein concentrations and incubation times. The fractions contained similar levels of 47 and 24 kDa proteins that reacted specifically with EP20 Ab on a Western blot. Type I elements expressed in MOPC 21 could also encode an integrase, but we would expect the protein to be larger than 47 kDa. We also tested the comparable fraction from mouse liver, which does not express IAPs, for endonuclease activity. High levels of activity were present, but no 47 or 24 kDa proteins were detected with EP20 Ab on Western blots. However, some larger cross-reacting protein was detected.

#### D. Translation of Type IIB Elements In Vitro

The structure of the type IIB elements determined by sequencing suggested they could encode a protein of 47 kDa if translation were to initiate at a unique methionine in ORF 2 introduced by duplication of a region of integrase. To test this, the type IIB element was transcribed and translated in vitro. The product contained a prominent 47 kDa protein, which, however, could not be immunoprecipitated with EP20 Ab. The in vitro product of a control cDNA known to have an ORF for a gag-pol fusion protein including the integrase was also not immunoprecipitated with EP20 Ab, although it was with a gag antibody. These results demonstrate that EP20 Ab cannot be used to precipitate integrase protein and are consistent with the lack of effect of the antiserum on the endonuclease activity.

Although the intracellular distribution and properties of the endonuclease activity we measured suggest that this may be a previously uncharacterized enzyme, inability to inhibit or complex the activity with EP20 Ab does not permit us to determine whether the activities detected in comparable fractions from two myelomas and liver result from expression of the same protein. We have also not been able to demonstrate a relationship between the endonuclease activity and the 47 kDa protein detected in myeloma cells with EP20 Ab. Thus, there is not evidence at this time that an integrase encoded by IIB elements plays a role in amplification of these IAP genes. This project will not be continued.

#### Publications:

Lueders K, Kuff E. Transposition of intracisternal A-particle genes, *Prog Nucleic Acid Res Mol Biol* 1989;36:173-86.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05265-07 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Regulation of Cytoskeletal Proteins

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

P. Wagner	Chemist	LB	NCI
N. D. Vu	Chemist	LB	NCI
Y. Wu	Visiting Fellow	LB	NCI
A. Rhoads	Guest Researcher	LB	NCI
A. Carroll	Biologist	LB	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Protein Biochemistry Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

4.5

## OTHER:

0

## CHECK APPROPRIATE BOXES)

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

(a1) Minors

(a2) Interviews

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Regulation of cytoplasmic myosins: Smooth muscle and nonmuscle myosins can be phosphorylated *in vivo* and *in vitro* by protein kinase C, PKC. Based on results with smooth muscle myosins, others have suggested that PKC phosphorylation of myosin inhibits contractile activity. To see if PKC might have a significant regulatory role in actomyosin-based cellular movements in nonmuscle cells, we examined the effect of PKC phosphorylation on a cytoplasmic myosin isolated from calf thymus. PKC phosphorylation had little effect on the actin-activated ATPase activity and filament stability of this myosin. These results indicate that PKC phosphorylation of cytoplasmic myosin does not directly regulate contractile activity.

Regulation of secretion: Norepinephrine secretion by permeabilized PC12 cells is ATP and  $Ca^{2+}$ -dependent. We have used ATP $\gamma$ S to examine the role of phosphorylation in the regulation of norepinephrine secretion from these permeabilized cells. While most kinases will use ATP $\gamma$ S to thiophosphorylate proteins, thiophosphorylated proteins are resistant to dethiophosphorylation by protein phosphatases. Our results indicate that  $Ca^{2+}$  regulates norepinephrine secretion in PC12 cells by activating a kinase. Once this kinase has phosphorylated its target protein, norepinephrine secretion is still ATP-dependent and no longer requires  $Ca^{2+}$ . Although this kinase is  $Ca^{2+}$ -dependent, it appears to be independent of calmodulin, and it does not appear to be protein kinase C. The role of GTP-binding proteins in the regulation of catecholamine secretion is also being investigated. As has been observed with other secretory cells, we find that GTP $\gamma$ S (or GMP-PNP) induces  $Ca^{2+}$ -independent norepinephrine secretion from permeabilized PC12 cells. AlF $_3$ , an activator of GTP-binding proteins, also caused about a 3-fold increase in  $Ca^{2+}$ -independent norepinephrine secretion. This suggests that the effect of GTP $\gamma$ S on secretion results from its binding to a GTP-binding protein.



## Project Description

### A. Regulation of Contraction

#### Objectives:

Our general goals are to understand the roles of cytoplasmic and smooth muscle actins and myosins, their interactions with each other and with the cell membrane, the regulation of these interactions, and how they relate to the various motile activities of cells and to the regulation of smooth muscle contraction.

#### Major Findings:

The 20,000-dalton light chains, LC20, of vertebrate smooth muscle and nonmuscle myosins are phosphorylated by myosin light chain kinase (MLCK). Phosphorylation by MLCK is thought to play a central role in the regulation of smooth muscle contraction and actomyosin-based cellular activities. In vitro, MLCK phosphorylation affects both the filament assembly and actin-activated ATPase activity of these myosins. Under conditions in which MLCK phosphorylation causes large increases in cytoplasmic myosin filament formation, it also causes large increases in the actin-activated ATPase activity. However, under conditions in which unphosphorylated cytoplasmic myosin is mostly filamentous, both phosphorylated and unphosphorylated myosins are active and MLCK phosphorylation has only a small effect on the actin-activated ATPase activity. Based on these results, we have suggested that phosphorylation might regulate actomyosin-based motile activities in vertebrate nonmuscle cells by regulating myosin filament assembly.

Smooth muscle and nonmuscle myosins may also be phosphorylated in vivo and in vitro by protein kinase C, a calcium, phospholipid-dependent protein kinase. PKC phosphorylation partially inhibits the actin-activated ATPase activity of MLCK-phosphorylated gizzard smooth muscle myosin but has no effect on the very low activity of the unphosphorylated myosin. Based on these results, other investigators have suggested that PKC phosphorylation might inhibit contractile activity. To see if PKC might have a significant regulatory role in actomyosin-based cellular movements in nonmuscle cells, we have examined the effect of PKC phosphorylation on filament formation and on the actin-activated ATPase activity of a cytoplasmic myosin isolated from calf thymus. PKC phosphorylation had little effect on the actin-activated ATPase activity and filament stability of calf thymus myosin. Under conditions where MLCK phosphorylation stabilizes filaments and increases ATPase activity, PKC phosphorylation does not reverse the effect of MLCK phosphorylation or mimic its effect on unphosphorylated myosin. Under conditions where unphosphorylated and MLCK-phosphorylated myosins are both filamentous, PKC phosphorylation neither destabilized the filaments nor inhibited the actin-activated ATPase activity. Thus, PKC phosphorylation of LC20 does not appear to directly regulate nonmuscle myosin filament formation or its interaction with actin.

B: Regulation of SecretionObjectives:

Secretion of neurotransmitters and hormones is usually triggered by an increase in cytoplasmic calcium. The mechanism(s) by which this rise in calcium induces secretion is unknown. The proteins and other molecules involved in the fusion of the secretory vesicles with the plasma membrane have not been identified, and the mechanism of this fusion is unknown. We are using permeabilized PC12 cells and bovine chromaffin cells to investigate roles of  $\text{Ca}^{2+}$ , ATP, GTP, and protein phosphorylation in secretion. This line of investigation may allow us to determine how  $\text{Ca}^{2+}$  induces secretion and to identify some of the proteins involved in the final steps of the secretory process.

Major Findings:

Rat pheochromocytoma PC12 cells have many of the characteristics of adrenal chromaffin cells. In response to  $\text{K}^+$ -induced depolarization PC12 cells secrete catecholamines in a  $\text{Ca}^{2+}$ -dependent manner. Exposure of PC12 cells to low concentrations of digitonin permeabilizes the plasma membrane but leaves the secretory vesicles intact. The release of catecholamines from these permeabilized cells is  $\text{Ca}^{2+}$  and ATP-dependent and occurs by exocytosis, i.e. fusion of the secretory vesicles with the plasma membrane. Permeabilization of the plasma membrane gives access to the cytoplasm and allows one to investigate the mechanism of secretion.

One possible mechanism by which a rise in intracellular  $\text{Ca}^{2+}$  could regulate secretion is protein phosphorylation by  $\text{Ca}^{2+}$ -dependent kinases. While stimulation of a variety of secretory cells has been shown to result in increased levels of protein phosphorylation, there is no direct evidence that these phosphorylations regulate secretion. We have used adenosine-5'-(3-thio)triphosphate, ATPYS, to investigate the role of phosphorylation in the regulation of norepinephrine secretion from permeabilized PC12 cells. While many kinases will use ATPYS to thiophosphorylate proteins, thiophosphorylated proteins are resistant to dethiophosphorylation and remain thiophosphorylated under conditions in which phosphorylated proteins are dephosphorylated.

Permeabilized PC12 cells were incubated with ATPYS for various lengths of time in the absence of  $\text{Ca}^{2+}$ . The ATPYS was removed, and the release of norepinephrine was determined in the presence and absence of  $\text{Ca}^{2+}$  and in the presence and absence of ATP. Preincubation with ATPYS increased the amount of norepinephrine secreted in the absence of  $\text{Ca}^{2+}$ , but it had no effect on the amount released in the presence of  $\text{Ca}^{2+}$ . After a 15 minute preincubation in 1 mM ATPYS, there was almost as much norepinephrine secretion in the absence of  $\text{Ca}^{2+}$  as in its presence. The effect of ATPYS appears to be specific because including ATP in the preincubations inhibited the effect of ATPYS. Secretion of norepinephrine from ATPYS-treated cells was still ATP dependent. This indicates that ATP participates in at least two steps in the secretory pathway. The rate of modification by ATPYS was  $\text{Ca}^{2+}$ -dependent as brief preincubations with ATPYS in the presence of  $\text{Ca}^{2+}$  resulted in higher levels of  $\text{Ca}^{2+}$ -independent secretion than did preincubations with ATPYS in the absence of  $\text{Ca}^{2+}$ . Similarly, brief preincubations with ATP in the presence of  $\text{Ca}^{2+}$  resulted in elevated levels of  $\text{Ca}^{2+}$ -

independent secretion. These results suggest that norepinephrine secretion by PC12 cells is regulated by a  $\text{Ca}^{2+}$ -dependent phosphorylation. Once this phosphorylation has occurred, norepinephrine secretion is still ATP-dependent but no longer requires  $\text{Ca}^{2+}$ . When permeabilized PC12 cells are incubated with ATP in absence of  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$ -independent phosphatases keep the level of phosphorylation low, and secretion does not occur. When the cells are incubated with ATPYS in the absence of  $\text{Ca}^{2+}$ , there is a slow build up of thiophosphorylated protein, and the cells secrete. When the cells are preincubated with ATPYS in  $\text{Ca}^{2+}$ , the kinase is activated resulting in higher levels of thiophosphorylation and higher subsequent levels of  $\text{Ca}^{2+}$ -independent norepinephrine secretion. Although this kinase is  $\text{Ca}^{2+}$ -dependent, it appears to be independent of calmodulin, and it does not appear to be protein kinase C. While this model is based primarily on the effects of ATPYS, the increased levels of  $\text{Ca}^{2+}$ -independent secretion from permeabilized cells preincubated in  $\text{Ca}^{2+}$  and ATP are consistent with norepinephrine secretion being regulated by a  $\text{Ca}^{2+}$ -dependent phosphorylation.

GTP-binding proteins have been implicated in the regulation of secretion both at the level of signal transduction and in some undefined way that occurs after signal transduction. As has been observed with other secretory cells, we have found that GTPYS (or GMP-PNP) induces  $\text{Ca}^{2+}$ -independent norepinephrine secretion from permeabilized PC12 cells. This  $\text{Ca}^{2+}$ -independent norepinephrine secretion is ATP-dependent. The level of norepinephrine secretion in the presence of  $\text{Ca}^{2+}$  is not affected by GTPYS. GTP and GDP $\beta$ S inhibit the effect of GTPYS on  $\text{Ca}^{2+}$ -independent norepinephrine secretion but have no effect on norepinephrine secretion in the presence of  $\text{Ca}^{2+}$ . The effect of GTPYS does not appear to be due to the release of  $\text{Ca}^{2+}$ , to the activation of protein kinase C, nor to the release of cAMP or cGMP. Presently, the mechanism by which GTPYS increases  $\text{Ca}^{2+}$ -independent secretion is unknown. The ability of these GTP analogs to induce  $\text{Ca}^{2+}$ -independent secretion suggests the involvement of a GTP-binding protein. Because  $\text{AlF}_3$  will activate GTP-binding proteins, we examined its effect on norepinephrine secretion in these permeabilized cells. The addition of 5  $\mu\text{M}$   $\text{AlF}_3$  and 5 mM  $\text{F}^-$  caused about a 3-fold increase in  $\text{Ca}^{2+}$ -independent norepinephrine secretion. This indicates that the effect of GTPYS on secretion results from its binding a GTP-binding protein. Extraction experiments indicate that this GTP-binding protein is on either the plasma membrane or the secretory vesicle membrane. We have found that the membrane of the secretory vesicle contains several GTP-binding proteins.

#### Publications:

Wagner PD, Vu ND. Filament assembly and regulation of the actin-activated ATPase activity of thymus myosin, *Biochemistry* 1988;27:6236-42.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05267-05 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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, Microbial Genetics and Biochemistry Section	LB	NCI
D. Chattoraj	Microbiologist	LB	NCI
K. Tilly	Sr. Staff Fellow	LB	NCI
K. Muraiso	Guest Researcher	LB	NCI
A. Ghosh	Visiting Scientist	LB	NCI
B. Funnell	Visiting Associate/Visiting Scientist	LB	NCI
E. Maguin	Visiting Fellow	LB	NCI
S. Dasgupta	Visiting Scientist	LB	NCI

## COOPERATING UNITS (if any)

Dr. S. Wickner, LMB, NCI; Dr. E.B. Hansen, Chr. Hansen's Laboratorium, Hørsholm, Denmark

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Microbial Genetics and Biochemistry Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

7.3

## PROFESSIONAL:

7.0

## OTHER:

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.)

Mechanisms that account for the stable inheritance of unit-copy plasmids continue to be the subject of our research. In the period of this report we have (1) demonstrated that the steric relationship between the origin of P1 plasmid replication and a replication control locus influences the efficiency of control. This result confirms our independent evidence for a physical interaction between the two loci; (2) more precisely mapped and characterized mutations within the repA gene of P1 that differentially affect the initiation function of RepA and a dominant inhibitory function of the repA gene that is expressed on overtranscription. The mutations were shown to incidentally alter other properties that are being studied in vivo and (in collaboration with S. Wickner) in vitro; (3) demonstrated that the lytic replicon of P1 is a participant in P1 plasmid maintenance; (4) mapped to rpoB one of several host mutations that were isolated as specifically deficient in miniP1 plasmid replication, thereby implicating a highly specialized role for RNA polymerase in the replication of this plasmid; (5) provided genetic evidence for roles of heat shock proteins DnaJ, DnaK, and GrpE in the plasmid replication of P1 and of the fertility factor F. This is the first evidence for the participation of these proteins in plasmid replication; (6) mapped and cloned one of two components of a P1 addictive system that assures the retention of P1 plasmid; (7) determined the extent to which host protein IHF and DNA supercoiling dramatically increase the affinity of the P1 partitioning protein ParB for the plasmid centromere, parS.



## Project Description

### Objectives:

We aim to elucidate mechanisms that (1) precisely control the initiation of DNA replication and (2) assure the stable maintenance of replicons by (a) appropriately partitioning them to daughter cells and (b) causing the death of cells from which the replicon has been lost. This year increasing emphasis has been placed on the participation of host proteins in these processes.

### Major Findings:

#### A. Position Effects on the Expression of a Replication Control Element

P1 plasmid replication is normally controlled by a locus, incA, composed of nine binding sites for the P1 initiator protein, RepA. With the recognition by Pal and Chattoraj (1988) that even a single binding site for the initiator protein can exert a significant inhibitory action on P1 plasmid replication, it has become possible to examine whether (and how) the orientation and distance between the plasmid origin and a discrete control locus affect the efficiency with which control is exerted. Dasgupta and Chattoraj have found that the efficiency of replication control is sensitive to both these variables. The plasmid copy number was lowest when the binding sites were inverted with respect to each other and on the same face of the DNA helix. These data provide independent evidence in support of the hypothesis of Chattoraj et al. (1988) that an interaction between DNA-bound RepA molecules mediates copy number control.

#### B. Characterization of Inhibitor-Resistant repA Mutants

Muraiso and Chattoraj have obtained several mutations in repA that inactivate the gene product for replication, but retain the capacity of the over-transcribed gene to inhibit replication of a wild type plasmid in trans. Mutations in repA that are insensitive to this second inhibitor have also been obtained. Of several such mutant miniP1 plasmids studied, one was shown to be characterized by a high copy number and partial incA-insensitivity, both in vivo and (by Wickner) in vitro. A remarkable property of this mutant is that it mediates the replication of a miniP1 that lacks the DnaA-binding sites normally essential to plasmid origin function, but the replication is nonetheless DnaA-dependent.

#### C. Participation of the Lytic Replicon in P1 Plasmid Maintenance

The lytic replicon of P1, previously characterized by Hansen (1989) - in part during tenure of a Fogarty fellowship in this laboratory - was shown by Yarmolinsky et al. (in press) to be a participant in P1 plasmid maintenance. P1 joins the increasing number of plasmids that are recognized as being composed of more than a single replicon.

#### D. Host Participation in P1 Plasmid Replication

The participation of E. coli heat shock proteins DnaK, DnaJ, and GrpE in miniP1 plasmid replication was shown in a series of genetic experiments by

Tilly and in a parallel series of biochemical experiments by Wickner. Genetic experiments with a miniF also performed by Tilly suggest that the replication of these similarly organized plasmids shows similar dependencies on DnaK, DnaJ, GrpE.

Ghosh and Chattoraj have isolated E. coli mutants that specifically impair P1 plasmid replication. One such mutant appears to carry an rpoB mutation, suggesting a highly specialized role for RNA polymerase in the replication of P1 as a plasmid.

#### E. Preliminary Genetic Analysis of Addictive Functions of P1

We previously noted that P1 plasmid loss causes cellular filamentation and death and that these symptoms of addiction could be prevented by particular  $\lambda$ -P1 chimeras. Yarmolinsky and Tilly have since identified a particular P1 EcoRI fragment to which the DNA of these chimeras hybridize. Clones of the region bearing the gene(s) that prevent host death (phd) in more versatile vectors than those originally employed have been constructed by Maguin. Isolation procedures by which to isolate the gene(s) responsible for death on curing (doc) are being developed.

#### F. Role of IHF and Supercoiling in ParB Binding to the Plasmid Centromere

Participation of the DNA-bending protein Integration Host Factor (IHF) in the binding of P1 partitioning protein ParB to the plasmid centromere, parS, was demonstrated last year by Funnell. This year she has undertaken to characterize the complexes of ParB with IHF and variant forms of parS. Her studies show that IHF and supercoiling each increases the affinity of ParB for parS by at least two or three orders of magnitude. Nevertheless, centromere activity is retained by variants of parS that cannot be stimulated by IHF. Despite a claim to the contrary, Funnell's evidence from competition experiments suggests that that IHF-insensitive variants of parS do not exhibit an altered specificity for subsequent partitioning.

#### Publications:

Yarmolinsky MB, Sternberg N. Bacteriophage P1. In: Calendar R, ed. The Bacteriophages, vol 1. 1988; New York: Plenum Press, pp. 291-438.

Pal SK, Chattoraj, DK. P1 plasmid replication: initiator sequestration is inadequate to explain control by initiator-binding sites, *J Bacteriol* 1988;170:3554-60.

Funnell BE. Participation of Escherichia coli integration host factor in the P1 plasmid partition system, *Proc Natl Acad Sci USA* 1988;85:6657-61.

Hansen EB. Structure and regulation of the lytic replicon of phage P1, *J Mol Biol* 1989;207:135-50.

Yarmolinsky, MB, Hansen EB, Jafri S, Chattoraj DK. Participation of the lytic replicon in P1 plasmid maintenance, *J Bacteriol* 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05268-02 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.)

Mechanisms of Meiotic Recombination

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

M. Lichten	Senior Staff Fellow	LB	NCI
T-C. Wu	Chemist	LB	NCI
C. Goyon	Visiting Fellow	LB	NCI
M. Daly	IRTA Fellow	LB	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Developmental Biochemistry and Genetics

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4

## PROFESSIONAL

4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unretarded type. Do not exceed the space provided.)

Our research is aimed at understanding the molecular mechanism of meiotic recombination in the yeast Saccharomyces cerevisiae and the means by which yeast exerts control over meiotic recombination. Techniques to detect heteroduplex DNA molecules formed during meiosis have been refined. These techniques have been used to determine the specificity of base-pair mismatch correction, the polarity of strand transfer during meiosis, and the timing of formation of heteroduplex DNA during meiosis. We have performed studies which address the roles that meiotic transcription and chromatin structure play in determining the level of meiotic recombination in a region. Finally, we have begun a search for sequences which modulate the level of meiotic recombination in their vicinity.

## Project Description

### Objectives:

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

We are also continuing our examination of the means by which control is exerted over the level of meiotic recombination in different parts of the genome and the gene products which participate in that control. We have completed an examination of the possible roles that transcription and chromatin structure have in regulating the level of meiotic recombination at different genomic locations, and are currently engaged in a search for "hot" and "cold" spots for meiotic recombination.

### Major Techniques Employed and Major Findings:

#### A. Detection of Heteroduplex DNA Products of Meiotic Recombination

In the previous report, we described the adaptation of the denaturant-gradient gel technique of Fischer and Lerman (Proc Natl Acad Sci USA 1983;80;1579) to allow detection of heteroduplex DNA molecules containing single base-pair mismatches. This modification allows the sensitive detection and resolution of the two mismatch configurations possible at a given site. We applied this methodology to examination of the meiotic behavior of a G > C transversion mutation in the ARG4 gene, and determined that only C/C mismatches escape mismatch correction and are found in spores. We were also able to deduce that the coding strand of the ARG4 gene was predominantly the one transferred to form heteroduplex DNA during meiotic recombination. However, limitations on the sensitivity of our assay made difficult a quantitative assessment of the biases in mismatch correction and strand transfer. Christophe Goyon has now refined the resolution and sensitivity of the above-described technique to the point where he can detect one heteroduplex molecule per 1000 haploid genomes. He is currently determining the timing of formation of heteroduplex DNA during meiosis; preliminary results indicate that heteroduplex molecules appear at very nearly the same time that cells become committed to carry out meiotic recombination. He is also examining the behavior of mutations in other yeast genes, to confirm the conclusions described above.

#### B. Analysis of Transcription and Chromatin Structure During Meiosis

In a large number of organisms, from yeast to man, different regions of the genome display widely different frequencies of meiotic recombination. In yeast, reporter genes inserted in a region display meiotic recombination frequencies commensurate with that observed for the region. Carol Wu and Michael Lichten have taken advantage of this finding to search for properties of regions displayed during meiosis which correlate with recombination activity. We initially examined the possibility that transcriptional



activity during meiosis was the sole determinant of the rate at which a gene recombined. We measured levels of transcript during meiosis of the LEU2 gene inserted at several loci; these insert loci displayed a greater than 30-fold range in levels of meiotic recombination; however, there was no difference between strains in the relative level of LEU2 mRNA at any time after initiation of sporulation. These findings appear to rule out transcription as a primary determinant of the level of recombination in a gene. We have extended this study to an examination of alterations in chromatin structure of the same inserts, initially at the level of nuclease hypersensitivity. Preliminary results indicate a lack of difference between "hot" and "cold" loci in their patterns of DNase I hypersensitivity.

### C. Sequences Which Modulate the Level of Meiotic Recombination

Michael Daly has initiated a search for sequences which either stimulate or repress meiotic recombination in their vicinity. This approach takes advantage of the fact that position effects are also observed in meiotic recombination between copies of leu2 present at different genomic locations (ectopic recombination). He has constructed strains with a deletion of the normal LEU2 locus that removes part of the coding region and a large region of 5' upstream sequences. He is creating a library of yeast DNA fragments in plasmids containing leu2 point mutants, which will be used to create a series of isogenic strains with the insert-containing plasmids inserted at either a "hot" or "cold" locus. In these strains, the frequency of Leu<sup>+</sup> recombinants is a function of the level of meiotic recombination at the insert locus. Strains are sporulated and screened for inserts which confer either a higher or lower frequency of Leu<sup>+</sup> recombinants. He has also developed a replicating screening procedure which allows rapid processing of large numbers of transformants. Preliminary experiments have supported the validity of this approach. Once transformants containing putative recombination modulators are obtained, the inserts will be recovered in *E. coli* by plasmid rescue, subcloned, and rescreened to localize sequences of interest.

#### Publications:

Lichten M, Haber JE. 1989. Position effects in ectopic and allelic mitotic recombination in Saccharomyces cerevisiae, *Genetics*; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05269-02 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

DNA Synthesis in Mammalian Cells: Studies of Nucleic Acid Binding Proteins

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

S. H. Wilson	Medical Officer	LB	NCI
A. Kumar	Expert	LB	NCI
P. Becerra	Guest Researcher	LB	NCI

## COOPERATING UNITS (if any)

H.R. Guy, NCI; K. Seamon, FDA; K. Williams, Yale; R. Karpel, University of Maryland; F. Cobianchi, CNR, Pavia, Italy; B. Kay, University of North Carolina

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Nucleic Acid Enzymology Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.3

## OTHER:

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.)

Physical biochemical studies of a single-stranded nucleic acid binding protein termed A1 were conducted. The protein binds cooperatively to either RNA or DNA and the full length protein binds much tighter than a truncated A1 protein lacking the glycine-rich COOH-terminal domain (residues 185-319). Our studies suggest the mechanism of A1 binding is, in some respects similar to that of two well-known prokaryotic ssDNA binding proteins: Binding involves charge-charge interactions and close approach of aromatic amino acids to nucleotide bases. Our findings suggest that both the NH<sub>2</sub>-terminal and COOH-terminal domains of the intact A1 protein make significant contributions to the overall free energy of binding of A1 to nucleic acids. cDNAs for Xenopus cognates for the mammalian A1 protein have been isolated and characterized.

Project DescriptionObjectives:

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

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

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

1. Immediately following transcription, heterogeneous nuclear RNA (hnRNP) becomes associated with a group of proteins to form a ribonucleoprotein (hnRNP) complex that is believed to function in the processing, stabilization and transport of unique sequence transcripts. Earlier, we cloned a cDNA for the A1 hnRNP protein and then over-produced the protein in E. coli. We now are using a variety of physicochemical approaches to identify functional domains in the protein. Sequencing revealed that the first 184 amino acids in A1 contain an internal repeat such that when residues 3-93 are aligned with 94-184, 33% of the amino acids are identical. Limited proteolysis and photochemical cross-linking experiments indicate that the internal repeats in A1 correspond to two independent nucleic acid binding domains.

With a view toward understanding structure-function relationships of a protein component of the eukaryotic heterogeneous ribonucleoprotein complex, we have studied the interactions of recombinant A1 protein, its N-terminal two-thirds (UP1), and C-terminal domain with the fluorescent polynucleotide, poly (ethenoadenylic acid), poly( $\epsilon$ A). The overall binding affinity of A1 for poly( $\epsilon$ A) is significantly greater than that of UP1. This can be partly, but not entirely explained by the existence of cooperativity in A1-nucleic acid interactions: The cooperativity parameter,  $\omega$ , for A1-poly( $\epsilon$ A) interaction is about 30, whereas UP1 binds nucleic acids non-cooperatively ( $\omega=1$ ). Gel retardation experiments performed with M13 DNA also show cooperative binding for intact A1 and non-cooperative binding for UP1. We now have found conditions for controlled proteolysis of A1 such that the COOH-terminal domain can be isolated in good yield. Binding experiments with the C-terminal domain show that this polypeptide is capable of binding to single-stranded nucleic acids. These results suggest that intact A1 has at least two potential binding regions for nucleic acids: one within the N-terminal domain and one within the C-terminal domain. Both intact A1 and the C-terminal domain are capable of protein-protein association. A synthetic peptide that is a polymer of the consensus sequence of the A1

C-terminal domain, GNFGGGRGGNYGGSRG (~10,000 daltons), binds poly( $\epsilon$ A) tenaciously and cooperatively ( $\omega$ -25). Competition titrations in the presence of other polynucleotides, e.g. poly(U), indicate that this polypeptide has a strong affinity for other nucleic acids, as well. Site-directed mutagenesis experiments are in progress to test the contribution of specific amino acid residues to the binding affinity.

2. We have identified the Xenopus cognates for the mammalian A1 protein. We isolated several Xenopus clones from oocyte, tailbud embryo, and leg muscle cDNA libraries and determined their nucleotide sequences. Except for insertions and deletions, the deduced Xenopus oocyte A1 protein sequence is 92% identical to the rat protein. The rat and Xenopus proteins are very similar at the secondary structure level as well. There are two segments in the COOH-terminal domain where deletions or insertions are apparent: the rat protein does not have a 48 residue sequence that is present in the frog protein, and the frog protein does not have a 12 residue sequence that is present in the rat protein. We have confirmed that the Xenopus oocyte A1 protein is larger than rat A1, by transcribing and translating the oocyte cDNA clones in vitro, followed by gel electrophoresis. A second isoform of the Xenopus A1 protein has been identified by examination of cDNA clones isolated from embryonic and adult leg muscle cDNA libraries. This second isoform has a different COOH terminus compared to the oocyte A1 form. Our findings suggest that both isoforms are encoded by 1-2 genes and are the result of alternative splicing that is developmentally regulated. We discuss the biological implications of alternative forms of the A1 component of heterogeneous nuclear ribonucleoprotein (hnRNP) particles.

#### Publications:

Jensen L, Kuff EL, Wilson SH, Steinberg A, Klinman D. Antibodies from patients and mice with autoimmune diseases react with recombinant hnRNP core protein A1, *J Autoimmunity* 1988;1:73-83.

Wilson SH: Mammalian hnRNP complex protein A1 and mechanism of single-stranded nucleic acid binding (Review). In: Wilson SH, ed. *Biosynthesis*. Caldwell, NJ, Telford Press; in press.

Trauger RJ, Talbott R, Culp P, Lipkin IA, Karpel R, Wilson SH, Elder, JH. Shared five amino acid epitope in endogenous retroviral GP70 and A1 hnRNP protein, *J Virol*; in press.

Kay BK, Sawhney R, Wilson SH. The primary structure and identification of isoforms of the A1 heterogeneous nuclear ribonucleoprotein of Xenopus laevis, *Proc Natl Acad Sci USA*; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05270-02 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

DNA Synthesis in Mammalian Cells: Mechanism of HIV Reverse Transcriptase

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

S. H. Wilson	Medical Officer	LB	NCI
J. Abbotts	IRTA Fellow	LB	NCI
C. Majumdar	Guest Researcher	LB	NCI
P. Kedar	Fogarty Fellow	LB	NCI
P. Becerra	Guest Researcher	LB	NCI
A. Kumar	Expert	LB	NCI

## COOPERATING UNITS (if any)

G. Zon and K. Seamon, FDA; S. Broder and J. Cohen, NCI; P. Torrence, NIH, T. Kunkel, NIH

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Nucleic Acid Enzymology Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.7

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	B
<input type="checkbox"/> (a1) Minors			
<input type="checkbox"/> (a2) Interviews			

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We continued detailed biochemical studies on mechanisms of action of mammalian DNA replication proteins. Steady-state kinetic analysis of the HIV reverse transcriptase revealed an overall kinetic scheme for the reverse transcriptase mechanism. In addition, properties of the first two steps in the proposed reaction pathway were obtained. A DNA segment containing the coding region for this enzyme was subcloned in an expression vector, and the enzyme was overproduced in E. coli. This recombinant enzyme is under study.

## Project Description

### Objectives:

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

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

### Major Findings:

#### A. Structure-Function Relationships of HIV Reverse Transcriptase

1. Enzyme-Primer Complex. A study of steady-state kinetics of polymerization by purified HIV DNA polymerase (reverse transcriptase) has been conducted. DNA synthesis was examined using a system of poly r(A) as template, oligo d(T) as primer, and dTTP as nucleotide substrate. The substrate initial velocity patterns point to an ordered mechanism with template primer adding first. This year, primer recognition by purified HIV reverse transcriptase has been investigated. Earlier we had found that the reaction pathway for DNA synthesis is ordered, with template-primer and free enzyme combining to form the first complex in the reaction sequence. We now find that d(C)<sub>28</sub> is a linear competitive inhibitor of DNA synthesis against poly[r(A)]-oligo[d(T)] as template-primer, indicating that d(C)<sub>28</sub> and the template-primer combine with the same form of the enzyme in the reaction scheme, i.e., the free enzyme. The phosphorothioate oligodeoxynucleotide Sd(C)<sub>28</sub> also is a linear competitive inhibitor against template-primer. However, the  $K_i$  for inhibition (~2.8 nM) is ~200-fold lower than the  $K_i$  for inhibition by d(C)<sub>28</sub>. Since the inhibition is linear competitive, the dissociation constant is equal to the  $K_i$  for inhibition. Filter binding assays confirmed high-affinity binding between Sd(C)<sub>28</sub> and the enzyme and yielded a  $K_D$  similar to the  $K_i$  for inhibition. Substrate kinetic studies of DNA synthesis using Sd(C)<sub>28</sub> as primer, and poly[r(I)] as template, revealed that the  $K_m$  for Sd(C)<sub>28</sub> is 24 nM. The  $K_m$  for this primer is, therefore, 8-fold higher than the  $K_D$  for enzyme-primer binding (2.8 nM). These results enable calculation of rate values for the enzyme-primer association ( $k_{on}=5.7 \times 10^8 M^{-1} s^{-1}$ ) and dissociation ( $k_{off}=1.6 s^{-1}$ ).
2. dNTP Recognition. Primer and dNTP recognition by purified HIV reverse transcriptase were further investigated. Earlier kinetic studies indicated that the reaction pathway for DNA synthesis is ordered, with template-primer and free enzyme combining to form the first complex in the reaction sequence (Majumdar et al., 1988). Through use of a substrate analogue for primer, further evidence was obtained for this first complex in the pathway, and rate values for enzyme-primer

association and dissociation were calculated. We found that 3'azido-dTTP is a linear competitive inhibitor of DNA synthesis against the substrate dNTP (dTTP) in the poly [r(A)]-oligo [d(T)] replication system. This indicates that 3'azido-dTTP and dTTP combine with the same form of the enzyme in the reaction scheme, i.e., the enzyme-primer complex. A second analogue, 3'amino-dTTP, also is an inhibitor against dTTP, but the mechanism is linear noncompetitive, indicating that dTTP and this inhibitor do not combine with the same form of the enzyme. Since the inhibition with 3'azido-dTTP is linear competitive, the dissociation constant is equal to the  $K_i$  for inhibition (20 nM). Next, substrate kinetic studies of DNA synthesis using 3'azido dTTP as substrate revealed that the  $K_m$  is 2 $\mu$ M. Therefore, the  $K_m$  for this substrate analogue is 100-fold higher than the  $K_D$  for binding to the enzyme-primer complex. This difference is consistent with the proposed kinetic scheme and is due to the effect of enzyme-primer complex dissociation on the value of  $K_m$ . These results enable calculation of real time rate values for the enzyme-oligo dT association ( $k_{on}=2.4 \times 10^8 M^{-1} s^{-1}$ ) and dissociation ( $k_{off}=35 s^{-1}$ ).

## B. Polymerase Error Mechanisms

1. We examined misincorporation by purified HIV-1 reverse transcriptase. The enzyme shows "bypass" synthesis in the absence of an individual dNTP substrate in a  $\phi$ X174 DNA template system. This bypass occurs during processive synthesis where the enzyme misincorporates and proceeds with replication rather than dropping off the template. In the presence of both dGTP and dTTP, the "error rate" for dGMP incorporation on poly rA template is 1/150. The results indicate that the HIV reverse transcriptase can tolerate certain base mismatches and could provide genetic diversity through error-prone replication. Tolerance of an A:G mispair is strong, such that with poly rA as template extensive processive poly dG synthesis is conducted. Enzyme processivity and polymerization rate for dGMP or dTMP incorporation are about the same, whereas the  $K_m$  for dGTP is approximately 1000-fold higher. Comparison of thermodynamic parameters for dGMP misincorporation and dTMP incorporation indicates different free energy of activation ( $\Delta G^*$ ) values, 13.6 and 9.2 kcal/mol, respectively, and a large difference in entropy of activation ( $\Delta S^*$ ); dTMP incorporation has a  $\Delta S^*$  of 73 cal/K mol, whereas  $\Delta S^*$  for dGMP misincorporation is -21 cal/K mol. These data suggest that a key differences in the mechanism between incorporation with A:T base pairing and incorporation with A:G mispairing in this system may be one exclusion of water molecules from the normal base pair and 2 G-G stacking interactions.
2. DNA-dependent DNA synthesis *in vitro* by HIV-1 reverse transcriptase (RT) is relatively error-prone. The enzyme, whether recombinant or from virus particles, produces errors while replicating M13mp2 DNA at a rate that, if operative *in vivo* would produce about five mutations per genome per round of replication. Sequence analysis of mutants resulting from *in vitro* synthesis demonstrates that the enzyme has unusual error specificity. Base substitution and one base frame shift mutational hotspots are observed. The specificity and position of errors suggest

that most of the frameshifts and many of the base substitutions are initiated by template-primer slippage. Processivity analysis for the enzyme on the M13mp2 DNA template reveals strong termination at specific sites. Termination sites within homopolymer sequences correlate with the frameshift mutational hot spots. The results suggest that the formation and/or utilization of misaligned template-primers is increased during the dissociation-reinitiation phase of the reaction.

Publications:

Majumdar C, Abbotts J, Roder S, Wilson SH. Studies on the mechanism of HIV DNA polymerase: steady-state kinetics processivity and polynucleotide inhibition, J Biol Chem 1988;263:15657-65.

Abbotts J, SenGupta DN, Zon G, Wilson SH. Studies on the mechanism of Escherichia coli DNA polymerase I large fragment: effect of template sequence and substrate variation on termination, J Biol Chem 1988;263:15094-103.

Majumdar C, Stein C, Cohen J, Broder S, Wilson SH: The stepwise mechanism of HIV reverse transcriptase: primer function of phosphorothioate oligodeoxynucleotide, Biochemistry 1989;28:1340-6.

Bebenek K, Abbotts J, Robert J, Wilson SH, Kunkel TA: Specificity and mechanism of error-prone replication by HIV reverse transcriptase, J Biol Chem; in press.

Abbott J, Wilson SH. Kinetics and thermodynamic parameters of misincorporation by HIV reverse transcriptase, J Biol Chem; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 CB 08212-15 LB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

From Gene to Protein: Structure, Function, and Control in Eukaryotic Cells

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

S. L. Berger	Research Chemist	LB	NCI
R. E. Manrow	Senior Staff Fellow	LB	NCI
M. S. Krug	Senior Staff Fellow	LB	NCI
A. R. Sburlati	Visiting Associate	LB	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBD

## SECTION

Genes and Gene Products Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

4.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews

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

The human prothymosin  $\alpha$  gene family has been cloned, isolated, and sequenced almost in its entirety. It consists of one functional gene, four processed pseudogenes, and one probable processed pseudogene. It is not known whether any of the pseudogenes is expressed; however, two of them contain open reading frames which encode proteins with 95 and 97% sequence homology when compared to one of the proteins encoded by the functional gene. The gene gives rise to two cDNAs by a unique form of alternate splicing. Functional studies of prothymosin  $\alpha$  cDNAs, mRNAs, cloned genes, transfected genes, and synthetic and natural proteins are not consistent with the proposed extracellular function of prothymosin  $\alpha$ . Prothymosin  $\alpha$  appears to be an intracellular protein which is required for cell division.

The mechanism of action of the ribonuclease H activity of reverse transcriptase from avian myeloblastosis virus and human immunodeficiency virus has been investigated. Contrary to previously held views, both ribonuclease H activities are endonucleases; they are apparently more sensitive to the conformation of the DNA:RNA substrate than the normal, cellular ribonuclease H activities. An inhibitor of the viral enzymes that has no effect on the normal ribonuclease H has been identified.

Project DescriptionProthymosin  $\alpha$ Objectives:

The function of prothymosin  $\alpha$  has been in question ever since we isolated a full length cDNA clone. Traditionally, prothymosin  $\alpha$  was believed to be a precursor for a series of related thymic hormone-like peptides. One of these, thymosin  $\alpha_1$ , is a highly acidic derivative containing the first 28 amino acids of prothymosin  $\alpha$ . It was believed to play a role in the immune system by stimulating immunoresponsiveness or immunocompetence in a variety of assays and by promoting the differentiation and maturation of immature thymocytes. Using an antibody to thymosin  $\alpha_1$ , the peptide could be detected at very low levels in the serum of normal individuals and at elevated levels in certain disease states, one of which was AIDS. In keeping with its function in the immune system, the peptide was shown to protect susceptible mice against opportunistic infections caused by Candida albicans. Thymosin  $\alpha_1$ , used therapeutically, was of limited value in the treatment of cancer, but provided symptomatic relief to immunosuppressed patients following irradiation: it was effective in improving the mixed lymphocyte response and in restoring or maintaining the correct ratio of helper to suppressor T cells. Treatment with thymosin  $\alpha_1$  did not increase the total number of circulating lymphocytes in these patients.

Further advances in thymic research did not support the prevailing view of thymosin  $\alpha_1$ . When analyzed under strictly denaturing conditions, the thymus contained no thymosin  $\alpha_1$ ; related sequences were found in prothymosin  $\alpha$ , a protein of greater than 110 amino acids. Enzymes capable of processing prothymosin  $\alpha$  into thymosin  $\alpha_1$  were conspicuously absent. Furthermore, at least part of what the antibody to thymosin  $\alpha_1$  recognized in sera was prothymosin  $\alpha$ . The precursor-product relationship seemed to be even more untenable when prothymosin  $\alpha$  performed better than thymosin  $\alpha_1$  in the mouse protection assay and in the lymphokine stimulation test. It seemed as though prothymosin  $\alpha$  was the active hormone.

Studies of prothymosin  $\alpha$  cDNAs, mRNAs, cloned genes, transfected genes, and synthetic and natural proteins carried out in this laboratory are not consistent with the proposed extracellular function of prothymosin  $\alpha$ . Two distinct cDNAs obtained in this laboratory did not contain nucleic acid sequences encoding an amino terminal signal peptide. Neither did they encode an internal hydrophobic region which might facilitate transport from the cell. Since an analysis of human DNA revealed a complex picture at the genomic level, it was possible that the isolated cDNAs represented an intracellular form of prothymosin  $\alpha$  and that a cDNA encoding a secreted form had not yet been discovered. Alternatively, prothymosin  $\alpha$  might be exclusively intracellular. Given these findings, it has become our aim to understand the role or roles played by prothymosin  $\alpha$  and prothymosin  $\alpha$ -like proteins regardless of their location in the cell.

Major Findings:

During the past year, we have completed the analysis of prothymosin  $\alpha$  genes which were cloned and partly characterized previously. We have now shown that

six genes account for all of the human genomic DNA fragments capable of hybridizing at high stringency with prothymosin  $\alpha$  cDNA. The six genes called 26, 28, 32, 34, 112, and 150 have been subcloned and sequenced with the exception of intron 1 of gene 28, and the extreme 3'-end of gene 150. The genes are approximately 90% homologous at the nucleic acid level.

Gene 28 meets all of the requirements for a functional gene. It has upstream regulatory elements including two CCAAT boxes at positions -115 and -83, respectively, and a TATA box at position -34. The TATATTAAA sequence is separated from the transcriptional start site (position 1) by a 24 base region with a GC content of 75%. The transcribed gene consists of 5 kb including 5 exons with sizes from 5' to 3' of 221, 72, 97, 74, and 731 base pairs, respectively. The nucleotide sequence of the exons matches that of the open reading frame in one cDNA perfectly, but differs from the sequence of the 5'- and 3'-noncoding regions at seven positions. The 4 introns are ~2600, 465, 459, and 285 base pairs, respectively. This gene apparently codes for two cDNAs by a variation of alternate splicing that has not been described before. The differential splicing mechanism affects the last triplet of intron 2 and first triplet of exon 3. Following a CT-rich region at the 3'-end of intron 2 are two GAG triplets: if the splice occurs immediately after the first AG sequence, the second GAG triplet becomes the first codon in exon 3 and results in the addition of a glutamic acid residue to the protein at position 40; if, on the contrary, the splice occurs after the second of the triplets in question, the GAG triplet remains within the intron and the glutamic acid residue is absent. This might be a general mechanism for manipulating a single amino acid, most probably a lysine (AAG) or a glutamic acid (GAG), in a protein. Since the glutamic acid-containing cDNA comes from fibroblasts, and the deficient cDNA comes either from spleen or lymphocytes, differential splicing may arise in a tissue specific manner.

Four of the five remaining genes have been classified as processed pseudogenes because they meet the following criteria: they differ both from each other and from gene 28 immediately upstream and downstream of the cDNA sequences; they lack introns; they have a poly(A) segment, sometimes degenerate, at the extreme 3'-end of the gene; and they each have a small, unique direct repeat of 13-16 base pairs immediately adjacent to the cDNA sequences. The latter is believed to occur upon insertion of the pseudogene into the genome. The fifth gene, 150, has no introns and a unique 5'-flanking region. However, owing to the absence of sequence information from the 3'-end, we cannot conclude that it, also, is a processed pseudogene. Gene 26 is the least conserved of these processed pseudogenes; it has an upstream initiation codon, deletions which cause frameshifts in the open reading frame (orf), and two copies of an Alu repetitive element inserted downstream of the orf. If expressed, it would code for a protein with minimum resemblance to prothymosin  $\alpha$ . Gene 32, with gene 150 in second place, is the most conserved. Their orfs have, respectively, 97 and 95% sequence homology at the amino acid level compared to human prothymosin  $\alpha$ , and they have consensus TATA boxes. Gene 34 and its allele, 35, and gene 112 encode aberrant proteins; gene 112 has 9 additional amino acids at the carboxyl terminus owing to loss of a stop codon, and genes 34 and 35, which display restriction fragment length polymorphism, diverge from the amino acid sequence of prothymosin  $\alpha$  following the alanine residue at position 86. We do not know whether any of the pseudogenes is expressed. However, if mRNAs for



genes 32, 34, 112, and 150 exist, they would be almost indistinguishable from that of gene 28 using size or hybridization behavior for characterization.

The structural analysis of the gene, pseudogenes and cDNAs does not support a role for prothymosin  $\alpha$  outside the cell.

Functional studies of prothymosin  $\alpha$  were also carried out. It is well known that mRNAs encoding proteins destined for export from the cell or for incorporation into the plasma membrane are translated on membrane-bound polysomes. Other mRNAs are translated on free polysomes. Therefore, we ascertained the location of prothymosin  $\alpha$  mRNA in myeloma cells and found it exclusively in the free polysome pool. These data suggest that prothymosin  $\alpha$  is not exported and that a cryptic signal peptide is highly unlikely. Our conclusions were supported by experiments with transfected cells. Despite the production of copious amounts of prothymosin  $\alpha$ , mammalian cells transiently transfected with the prothymosin  $\alpha$  gene did not secrete the encoded protein. In this context, it may be significant that the protein contains a consensus nuclear translocation signal.

As a result of these studies we favor an intracellular function for prothymosin  $\alpha$  that is essential for normal cell growth. Evidence for this point of view has recently been obtained from experiments with prothymosin  $\alpha$ -deficient cells. Under these conditions, abnormalities in cell division were observed.

#### Ribonuclease H

##### Objectives:

The mechanism of the ribonuclease H (RNase H) activity of reverse transcriptase has been investigated with the aim of finding specific inhibitors. According to the literature, the RNase H activity of reverse transcriptases from several sources was believed to be exonucleolytic whereas normal, cellular RNase H activities were shown to be endonucleolytic. These observations were based on the failure of the viral enzymes to cleave three substrates for the normal enzyme from Escherichia coli. The first of these was linear poly(A):poly(dT) in which the 5'-end of the RNA moiety was coupled to cellulose and the 3'-end was modified by the addition of a poly(C) tail. With both ends blocked in this manner, the viral enzyme from avian myeloblastosis virus was unable to cleave the RNA strand. However, when either end was not blocked, cleavage of the hybrid was successful. The requirement of the viral enzyme for a terminus, either 5' or 3', was confirmed by experiments with two circular substrates: poly(A) hybridized to poly(dT); and supercoiled plasmid DNA with ribonucleotide substitutions. Whereas E. coli RNase H digested the hybridized, circular RNA and relaxed supercoiled molecules, no viral enzyme affected either molecule. We thought that these differences could be exploited in designing inhibitors.

##### Major Findings:

Based on experiments performed in this laboratory, a novel view of the enzyme has emerged. In order to approach the problem of mechanism differently, we used a new substrate, globin mRNA labeled at the 3'-terminus with one residue of [ $^{32}\text{P}$ ]adenosine. This substrate, in the presence of oligo(dT), allowed



measurements of the deadenylation of globin, a much more sensitive assay than those used previously. Blocked substrates were also constructed using labeled globin mRNA as the starting material. Since globin mRNA is protected at the 5'-end by the naturally occurring cap structure, our attention was focused on the 3'-end. Fully blocked RNA was prepared by one of the following methods: ligation of pCp; ligation of an oligo(dT) tail; and polymerization of a long covalently attached complementary DNA tail onto the 3'-hydroxyl group of RNA. In each case, regardless of the 3'-end modification employed, globin mRNA was deadenylated at the same rate as the unmodified, labeled control in the presence of oligothymidylate. The data suggested that reverse transcriptase exhibited extremely broad specificity with respect to termini or that the RNase H activity of reverse transcriptase was an endonuclease.

To prove that the viral enzymes were indeed endonucleases, we synthesized three large, flexible, circular substrates. Two of them had an extended heteroduplex region in order to insure that at least a segment of the DNA:RNA hybrid would be able to assume the conformation of linear hybrids in solution. This was not the case with the circular substrates used in earlier studies. The newly synthesized circles were subsequently purified, characterized, and tested with three different RNase H enzymes. The most technically demanding is called "ligated hybrid". It consists of a relaxed, covalently closed circular DNA in which 770 consecutive bases are ribonucleotides. Ligated hybrids were separated from "unligated hybrids", the identical molecule with nicks at either end of the RNA moiety, by treatment with endonuclease free DNA polymerase I. Molecules that contained nicks or gaps were completely digested by the exonuclease activities of this enzyme. The ligated hybrids were evaluated with exonuclease III, mung bean nuclease, DNase and RNase. Taken together, the data showed that the ligated hybrid was a perfectly base paired circular molecule without defects. When both the ligated and unligated hybrids were tested as substrates with E. coli RNase H, the RNase H activity of AMV reverse transcriptase and a recombinant reverse transcriptase from HIV, all three enzymes could degrade both substrates, essentially at the same rate. Our interpretation was that all the enzymes are endonucleases and that that conformation, rather than lack of termini, caused other substrates to fail when tested with viral enzymes.

Using a third circular substrate, "bandaid", which consists of a large circular DNA molecule hybridized to a 770-base pair synthetic RNA, we have shown that ribonucleoside-vanadyl complexes inhibit the viral RNase H activities but not the normal enzyme from E. coli. This observation may prove to be important in the design of drugs for the treatment of AIDS.

#### Publications:

Eschenfeldt WH, Manrow RE, Krug MS, Berger SL. Isolation and partial sequencing of the human prothymosin  $\alpha$  gene family: evidence against export of the gene products. *J Biol Chem* 1989;264:7546-55.

Krug MS, Berger SL. Ribonuclease H activities associated with viral reverse transcriptases are endonucleases. *Proc Natl Acad Sci USA* 1989;86:3539-43.

## SUMMARY STATEMENT

### ANNUAL REPORT LABORATORY OF CELLULAR ONCOLOGY DCBD, NCI

October 1, 1988 through September 30, 1989

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. They also elucidate structure-function correlations through detailed examination of individual genes which have been implicated in neoplasia. Spontaneous tumors from humans and other species are examined for the presence of exogenous genes or altered cellular genes. The main research results for the past year are as follows:

#### Tumor gene expression *in vitro* and *in vivo*

Oncogene studies have involved *ras* encoded proteins and the EGF receptor. A major problem of *ras* biology is to determine how the protein is stimulated to become biologically active and to identify the target(s) with which *ras* proteins interact. In collaboration with B. Willumsen, we have identified an effector region that apparently interacts with the putative *ras* target. In collaboration with F. McCormick and B. Willumsen, we found that a cytoplasmic protein called GAP (for GTPase activating protein) interacts with the *ras* effector domain. This result raises the possibility that GAP may be a *ras* target, although the ability of GAP to inactivate normal *ras* encoded protein suggests that GAP may be only a negative regulator of *ras*. To distinguish between these two possibilities, we have constructed a series of mutants (in collaboration with J. Stone) within the effector domain to determine if GAP stimulation and transformation activity are coordinately affected. Mutants with wild type transforming activity, reduced transforming activity, and defective in transformation have been identified. While the GAP activity of some mutants was similarly reduced, a lack of correlation between the two activities has been noted for several mutants. These results suggest GAP may function principally as a negative regulator of *ras* and may not be the *ras* target.

In EGF receptor studies, we have previously demonstrated that when *hEGFR* encoding the normal human EGF receptor was overexpressed in established mouse NIH 3T3 cells, they developed a fully transformed phenotype that required ligand. Using this sensitive bioassay, we have now found that the C-terminus of the human EGF receptor facilitates proliferation of NIH 3T3 cells. Mutants which encode receptors lacking the last one or two C-terminal tyrosines of the receptor resulted in mutant receptors whose biological activity was lower than that of the full length receptor. These results are the opposite of those obtained previously for two other tyrosine kinases (the CSF-1 receptor [*c-fms*] and *c-src*), where removal of their C-terminal tyrosine increases their biological activity. We conclude the C-terminus of tyrosine kinases are functionally heterogeneous.

#### Analysis of Papillomaviruses

The strong association between certain papillomavirus (PV) types and specific human cancers, in particular cervical cancer, has led us to investigate the biological activities of papillomavirus genes and the biochemical activities of their encoded proteins. Bovine (B)PV has served as a model for studying papillomaviruses because the viral genome readily transforms and replicates autonomously in rodent cell lines. E5 is one of the two major transforming genes of BPV.

E5 encodes a 44 amino acid protein that is associated with the external surface of cytoplasmic membranes. Given its small size and location, it seemed unlikely that E5 would have intrinsic enzymatic activity, and we have therefore investigated the possibility that it induces transformation by activating other proteins involved in cell proliferation and transformation. We have determined that E5 can potentiate the transforming activity of two growth factor receptors, the *c-erbB* and *c-fms* proto-oncogenes but not two other tyrosine kinases, *c-src* or *c-fes*. Potentiation can occur in either the presence or absence of exogenous ligand. In E5 containing cells, the EGF receptor is partially activated in the absence of exogenous ligand, and the half life of both ligand stimulated and unstimulated receptor is increased. From these results, we propose that E5 induces transformation by inhibiting the down regulation of activated growth factor receptors.

Since the HPV16 E6 and E7 genes are selectively retained and expressed in HPV16 and HPV 18 associated cervical carcinomas, we have undertaken an in vitro analysis of their function and products. Biochemically, we found both E6 and E7 of HPV 18 and of BPV can bind zinc, presumably through conserved cys-x-x-cys motifs. In BPV E6, we have found that these motifs are essential for biological activity and for normal sub-cellular localization of the protein to the nuclear matrix. For HPV 16, E7, but not E6, was found to transform NIH3T3 cells. E7 could also extend the lifespan of normal human foreskin keratinocytes, but E6 was required in addition for immortalization of the cultured keratinocytes. The results with human keratinocytes provide strong support for the hypothesis that the preferential maintenance and expression of E6 and E7 in human cervical carcinomas has pathogenic significance.

In clinical studies, the smoke plume from bovine and human warts treated by laser or electrocoagulation was found to contain potentially infectious virus. An ordinary surgical mask was found to block this material, suggesting this simple means may eliminate the potential biohazard for operators.

### Biology of *fps/fes* oncogenes

This project aims to elucidate the biological functions(s) of the human *c-fps/fes* proto-oncogene and to understand the molecular basis of its oncogenic potential. Using efficient retroviral vectors, we have shown for the first time that when normal human *c-fps/fes* is expressed at sufficiently high levels, it can cause tumorigenic transformation of mouse cells. In addition to level of expression, the transforming activity of *c-fps/fes* can be modulated at a second level, by incubation of cells overexpressing the *c-fps/fes* product NCP92 with low concentration of a phosphatase inhibitor (sodium vanadate). This treatment potentiated the transforming activity of *c-fps/fes* by two orders of magnitude in a reversible, dose-dependent manner. The potentiating effect of vanadate was found to be relatively specific for *c-fps/fes*. Biochemical analysis of *c-fps/fes* transformed cells indicated that the potentiating effect of vanadate was not secondary to an increase in the specific activity of NCP92 kinase. The data were consistent with the hypothesis that the increase had resulted from the stabilization of phosphotyrosine in putative critical targets of NCP92.

### Retrovirus replication and cellular oncogene expression

The polymerase chain reaction (PCR) has been applied to 6 DNA samples from seropositive Taiwanese subjects to detect HIV-1 proviral sequences. The results suggest that these 6 samples contain various numbers of HIV-1 proviral copies which exhibit heterogeneity in their *env* sequences and are divergent from those of prototype HIV-1. Sequence analysis of two DNA clones, obtained by PCR-amplification of DNA from an asymptomatic homosexual, revealed the presence of an HIV-1 *env*-related sequence of 81 nucleotides containing 3 substitutions that were flanked by about 400 bp nucleotides of unknown origin. This interesting finding suggests that the



provirus may be defective and integrated in cellular sequence, possibly adjacent to human an endogenous retroviral sequence. The biological and clinical significance of this phenomenon needs further investigation.

Screening of a cDNA library for a possible oncogene associated with the spontaneous reticulum cell neoplasm (RCN) of SJL/J mice resulted in several cDNA clones which hybridized with the RNAs of RCN more intensely than with those of normal lymphoid tissues. These cDNA clones were sequenced, and a homology search revealed highly repetitive LTR sequences which were derived from endogenous murine retroviruses. The presence of a flanking sequence which is partially homologous to an IL-2-like lymphokine sequence suggests that overproduction of this putative lymphokine-like substance by RCN may participate in the pathogenesis of RCN in SJL/J mice.

### Genetic mechanism of neoplastic transformation

The thrust of this study is to elucidate the genetic mechanism of neoplastic transformation of normal tissues. The experimental system focuses on a 3.1 kb moderately cell-transforming human oncogene, hhcM, isolated from an African hepatocellular carcinoma cell line, Mahlavu. Determination of the nucleotide sequence of hhcM has now been completed. The hhcM nucleotide sequence shares no significant homology with other known oncogenes. It predicts an uninterrupted open reading frame specifying a message for a polypeptide of 467 amino acids, ca 52 kD. This was supported by the observation of a 52-55 kD polypeptide obtained in hybrid-select cell-free translation studies. This ORF was used in a chimeric construct with the lacZ promoter sequence, and production of the 52 kD protein has been confirmed in this construction, which also permits large scale production of chimeric protein. The chimeric protein is being used to generate antibodies to be employed in characterization of the protein in cultured cells as well as in clinical samples of human urine, serum, and liver.

### Role of protein kinases in modulating cell growth and malignant transformation

The focus of this project is to better elucidate the possible involvement of transmembrane signal transmission systems in the regulation of cell growth and as possible targets in the process of tumor initiation and tumor promotion. Studies carried out to better elucidate the regulatory properties of protein kinase C (PKC) have established that this enzyme is markedly influenced by oxidative modification. Under mild conditions, treatment of the isolated enzyme or of intact cells with agents which generate reactive oxygen species leads to direct conversion of PKC to an activated form which is no longer dependent upon lipids. These results suggest that the interrelationship between the generation of oxygen free radicals and modulation of PKC may play a significant role in the mechanism of chemical carcinogenesis and tumor promotion.

Since PKC plays a major role in cell growth regulation and tumor promotion, studies have been aimed at identifying proteins and activities modified by PKC to in turn transduce a signal to other components of the cell, including the nucleus. One activity found to be modified by initial activation of PKC and which may play an important role in the signal transduction cascade is the serine/threonine protein kinase encoded by the raf proto-oncogene. Collaborative studies with Dr. U. Rapp suggest that raf protein kinase may be directly activated by PKC. Further, we have observed that treatment of growth arrested NIH 3T3 cells with the mitogen PDGF or with phorbol ester tumor promoter provokes a rapid redistribution and activation of the raf protein kinase to the perinuclear region. The data suggest that a cascade may be involved in the transmission of mitogenic signal from the plasma membrane through PKC to activation of the raf kinase at the nucleus.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03663-13 LCO

## PERIOD COVERED

October 1, 1988 through September 30, 1989

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

Tumor gene expression *in vitro* and *in vivo*

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

PI:	D. R. Lowy	Chief, Lab Cellular Oncology	LCO NCI
OTHER:	T. J. Velu	Visiting Associate	LCO NCI
	K. Zhang	Visiting Fellow	LCO NCI
	W. D. Ju	Biotechnology Fellow	LCO NCI
	A. G. Papageorge	Microbiologist	LCO NCI
	W. C. Vass	Biologist	LCO NCI

## COOPERATING UNITS (if any)

See next page.

## LAB/BRANCH

Laboratory of Cellular Oncology

## SECTION

## INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.1

## PROFESSIONAL:

3.6

## OTHER:

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.)

Oncogene studies have involved *ras* encoded proteins and the EGF receptor. A series of *in vitro* and *in vivo* analyses of variants of Harvey murine sarcoma virus (Ha-MuSV), whose viral oncogene is a *ras* gene activated by two missense mutations, was carried out. We found the biological activity of Ha-MuSV was influenced significantly by an enhancer sequence located outside the LTR as well as by coding sequences, the biological level of *ras* affected the tumor target tissue of Ha-MuSV, and the *in vivo* pathogenicity of the Ha-MuSV variants correlated with their transforming activity on NIH 3T3 cells.

The GAP protein (GTPase activating protein), which can accelerate the intrinsic GTPase activity of normal *ras* (thus inactivating the protein), but not of highly transforming mutated versions of *ras* (thus permitting them to remain active), has been found to interact with the *ras* effector domain. This result raises the possibility that GAP may be a *ras* target, although the ability GAP to inactivate normal *ras* suggests GAP may function principally as a negative regulator of *ras*. To distinguish between these two possibilities, a series of mutants within the effector domain have been analyzed to determine if GAP stimulation and transformation activity are coordinately affected. A lack of correlation between the two activities has been noted for several mutants, which suggests GAP may function principally as a negative regulator of *ras* and may not be the *ras* target.

In EGF receptor studies, we have found that the C-terminus of the human EGF receptor facilitates proliferation of NIH 3T3 cells. Mutants which encode receptors lacking the last one or two C-terminal tyrosines of the receptor resulted in mutant receptors whose biological activity was lower than that of the full length receptor. These results are the opposite of those reported previously for two other tyrosine kinases (the CSF-1 receptor [*c-fms*] and *c-src*). They therefore indicate functional heterogeneity for the C-terminus of tyrosine kinases.

Cooperating Units:

LBP, NCI, FCRF, Dr. H.-F. Kung  
 Jackson Laboratory, Bar Harbor, Maine, Dr. J. Stone  
 Cochin Hospital, Paris, France, Dr. P. Tambourin  
 University Microbiology Institute, Copenhagen, Denmark, Drs. B. Willumsen and L. Beguinot.  
 University of Nice, Nice, France, Dr. J. Pouysségur.  
 Hebrew University of Jerusalem, Jerusalem, Israel, Dr. A. Levitzki.  
 Cetus Corporation, Emeryville, CA, Dr. F. McCormick

Major Findings:

1. *ras* oncogenes. We have used Harvey murine sarcoma virus (Ha-MuSV), whose encoded transforming *ras* protein p21 differs from its normal cellular proto-oncogene at only 2 amino acids. We have studied the contribution of coding and noncoding viral sequences to the biological activity of Ha-MuSV, using focal transformation of mouse NIH 3T3 cells in vitro and tumorigenicity of injected virus in vivo. A Ha-MuSV variant that encoded the proto-oncogene protein induced lymphoid leukemias (with thymomas), with a relatively long latent period, rather than the fibrosarcomas and erythroleukemias characteristic of Ha-MuSV with one or both missense mutations. An enhancer activity was identified in a (VL-30 derived) viral segment located outside the *ras* gene and outside the viral LTR. A variant Ha-MuSV from which this enhancer containing non-coding segment was deleted expressed lower levels of the wild-type viral protein, displayed impaired transforming activity in vitro, and induced lymphoid leukemias in vivo (with thymomas). It was concluded: 1) the biological activity of Ha-MuSV can be influenced significantly by noncoding sequences located outside the LTR as well as by coding sequences. 2) some sequences outside *ras* and the LTR positively regulate the expression of the viral *ras*, 3) relatively low biological levels of *ras*, whether resulting from low-level expression of wild type viral *ras* or high-levels of *ras* proto-oncogene protein, induce a type of tumor that differs from tumors induced by high biological levels of *ras*, and 4) the in vivo pathogenicity of the Ha-MuSV variants correlated with their transforming activity on NIH 3T3 cells. In collaboration with E. Gelmann, the selective advantage of both missense mutations over either one alone was shown by finding that only wild type viral *ras* (with both mutations) was able to bypass the dependence of MCF-7 human breast cancer cells upon estrogen for tumor formation.

A major problem of *ras* biology is to determine how the protein is stimulated to become biologically active and to identify the target(s) with which *ras* proteins interact. In collaboration with B. Willumsen, we have identified an effector region (amino acids 31-42) that apparently interacts with the putative *ras* target. This region is identical in proto-oncogene encoded *ras* proteins and in highly oncogenic versions of *ras*, which suggests that all *ras* proteins interact with the same target. In collaboration with F. McCormick and B. Willumsen, we found that a cytoplasmic protein called GAP (for GTPase activating protein), which can accelerate the intrinsic GTPase activity of normal *ras* (thus inactivating the protein), but not of highly transforming mutated versions of *ras* (thus permitting them to remain active), interacts with the *ras* effector domain. This result raises the possibility that GAP may be a *ras* target, although the ability GAP to inactivate normal *ras* encoded protein suggests, rather, that GAP may function principally as a negative regulator of *ras*. To distinguish between these two possibilities, we have constructed a series of mutants (in collaboration with J. Stone) within the effector domain to determine if GAP stimulation and transformation activity are coordinately affected. Mutants with wild type transforming activity, reduced transforming activity, and defective in transformation have been identified. While the GAP activity of some mutants was similarly reduced, a lack of correlation between the two activities has been

noted for several mutants. These results suggest GAP may function principally as a negative regulator of *ras* and may not be the *ras* target.

2. EGF receptors. A potential role for the normal EGF receptor in tumor formation has been proposed by others from the observation that increased numbers of apparently normal EGF receptors may be present in various human tumors and tumor cell lines. Consistent with this hypothesis, we have previously demonstrated that when *HEGFR* encoding the normal human EGF receptor was overexpressed in established mouse NIH 3T3 cells, they developed a fully transformed phenotype that required ligand. In NIH 3T3 cells expressing the human receptors, we have now found (in collaboration with L. Beguinot) that ligand stimulation rapidly (15 seconds) induces polyphosphoinositide hydrolysis and an increase in cytosolic calcium, secondary to release of intracellular calcium stores, followed by an increase in intracellular pH. Overexpression of the receptors has also been used to develop a highly sensitive bioassay that is specific for EGF receptor ligands. In this system, the cells can be grown in serum free medium with EGF as the only growth factor. In collaboration with J. Pousségur, overexpression of human EGF receptors in a hamster cell line (CCL39) conferred an EGF dependent mitogenic response, increased phosphoinositol turnover, and stimulated sodium/hydrogen ion exchange. Although the CCL39 cells can be transformed to tumorigenicity by an activated *ras* gene, EGF treatment of the hamster cells that overexpressed the human EGF receptors did not induce morphological transformation, despite expressing even higher numbers of receptors than NIH 3T3 cells which can be transformed by EGF.

In collaboration with L. Beguinot, we have also studied the consequences of shortening the C-terminus of the human EGF receptor by introducing premature terminations near the 3' end of the coding sequence. These deletions were carried out to determine if the C-terminus of the EGF receptor functioned in a manner similar to that of the CSF-1 receptor (*c-fms*) and *c-src*, two tyrosine kinases in which removal of the C-terminal tyrosine and the downstream amino acids increases the biological activity of the proteins. Furthermore, the v-erbB proteins of the avian erythroblastosis virus (AEV) lack the last one or two tyrosine residues, depending upon the strain of AEV, as well as being truncated in their N-terminus. In contrast to results obtained with *c-fms* and *c-src*, we found that the C-terminus of the human EGF receptor facilitates cell proliferation. Mutants which encode receptors lacking the last one or two C-terminal tyrosines, created by introducing premature terminations that delete the last 19 or 63 amino acids of the receptor, respectively, resulted in proteins whose biological activity was lower than that of the full length receptor. These results indicate an important functional heterogeneity for the C-terminus of tyrosine kinases.

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Velu, T.J., Vass, W.C., Lowy, D.R., and Tambourin, P.E. Harvey murine sarcoma virus: influence of coding and noncoding sequences on cell transformation in vitro and oncogenicity in vivo. *J. Virol.*, 1989; 63: 1384-1392.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER  
**Z01 CB 09052-01 LCO**

PERIOD COVERED  
**October 1, 1988 through September 30, 1989**

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

**Analysis of Papillomaviruses**

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

PI:	J. T. Schiller	Senior Staff Fellow	LCO NCI
OTHER:	D. R. Lowy	Chief	LCO NCI
	P. Hawley-Nelson	Biotechnology Fellow	LCO NCI
	M. S. Barbosa	IRTA Fellow	LCO NCI
	P. Martin	Visiting Fellow	LCO NCI
	N. L. Hubbert	Microbiologist	LCO NCI
	W. C. Vass	Biologist	LCO NCI
	M.A. Pritting	Chemist	LCO NCI

COOPERATING UNITS (if any)

See next page

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:

6.5

PROFESSIONAL:

4.0

OTHER:

2.5

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

B

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The BPV ORFs E5 and E6 encode proteins that independently transform rodent cells and ORF E1 and E2 encode proteins that indirectly effect the transforming activity of the virus. BPV E5 was shown to potentiate the transforming activity of transfected growth factor receptors in a ligand independent manner. Analysis of the receptor processing suggests that E5 transforms cells by retarding the down regulation of activated receptors. Mutational analysis of BPV E6 indicated that the conserved cys-x-x-cys motifs of the protein and its association with the nuclear matrix are require for its transforming activity.

We have identified a series of mutants in the E1 ORF that have higher transforming activity and transcription rates than the wild type viral genome suggesting that an E1 gene product suppresses expression of the E6 and/or E5 transforming genes. The three previously identified BPV E2 proteins have been assigned to specific viral transcripts and functionally analyzed with respect to transcriptional trans-activation, trans-repression, sequence specific DNA binding and dimer formation. BPV E2, and also E5, was shown to cooperate with BPV E6 in the immortalization of rat embryo fibroblasts. Consistent with these results indicating a direct effect of E2 on cell physiology, the induction of specific cellular proteins by E2 was demonstrated by 2-D gel analysis.

Since the HPV16 E6 and E7 genes are selectively retained and expressed in HPV16 associated cervical carcinomas, we have undertaken an in vitro analysis of their products. E7, but not E6, transformed NIH3T3 cells. E7 could also extend the lifespan of normal human foreskin keratinocytes, but E6, in addition, was required for immortalization of the cultured keratinocytes. Both E6 and E7 were shown to bind zinc, presumably through conserved cys-x-x-cys motifs. In clinical studies, the smoke plume from bovine and human warts treated by laser or electrocoagulation was found to contain potentially infectious virus; an ordinary surgical mask was found to block this material.

Cooperating Units:

LTVB, NCI, Drs. P. Lambert and P. Howley.

LB, NCI, Drs. J. Doniger and J. DiPaolo.

Tufts New England Medical Center, Boston, Massachusetts, Dr. E. J. Androphy.

University of Iowa School of Medicine, Iowa City, Iowa, Dr. L. Turek.

University of Rochester School of Medicine, Rochester, New York, Dr. D. Young

University of Pennsylvania, Drs. L. M. Dzubow and P. J. Weber.

Ludwig Institute for Cancer Research, St. Mary's Hospital Medical School, London,  
United Kingdom, Dr. K. H. Vousden.

University of Nice, Nice, France, Dr. F. Cuzin.

University of Erlangen, West Germany, Dr. H. Pfister.

Major Findings:

1. BPV E6 gene. The strong association of certain papillomavirus types with certain human cancers, in particular cervical cancer, has led us to investigate the biological activities of papillomavirus genes and the biochemical activities of their encoded proteins. BPV has served as a model for studying this group of viruses because the viral genome readily transforms and replicates autonomously in rodent cell lines. In previous studies we have determined that two BPV genes, E5 and E6, can independently induce morphologic transformation. We are currently investigating the mechanisms through which the E5 and E6 protein induce cellular transformation. A mutational analysis of the E6 gene (in collaboration with Karen Vousden and Elliot Androphy) has revealed that the four conserved *cys-x-x-cys* motifs, as well as several other amino acids, are critical for the transforming activity of the protein. Preliminary subcellular localization of the mutant proteins has suggested that association with the nuclear matrix may be required for transformation.

2. BPV E5. BPV E5 encodes a small 44 amino acid protein that is associated with the external surface of cytoplasmic membranes. Given its small size and location, it seemed unlikely that E5 would have intrinsic enzymatic activity, and we have therefore investigated the possibility that it induces transformation by activating other proteins involved in cell proliferation and transformation. We have determined that E5 can potentiate the transforming activity of two growth factor receptors, the *c-erbB* and *c-fms* proto-oncogenes but not two other tyrosine kinases, *c-src* or *c-fes*. Potentiation can occur in either the presence or absence of exogenous ligand. In E5 containing cells, the EGF receptor is partially activated in the absence of exogenous ligand, and the half life of both ligand stimulated and unstimulated receptor is increased. From these results, we propose that E5 induces transformation by inhibiting the down regulation of activated growth factor receptors.

3. BPV E2. The BPV E2 ORF is an important regulator of viral gene expression, via the ability of its products to trans-activate and trans-repress; both functions have been genetically mapped to the E2 ORF. We have previously determined the E2 ORF encodes three distinct proteins that bind specific conserved sequences in the viral genome. In collaboration with Paul Lambert and Peter Howley, we have mapped each protein species to a specific transcript and so have determined that the 31 kd and 28 kd proteins, containing only C terminal E2 peptides, are trans-repressors and the full-length 48 kd protein is a trans-activator. We have also completed a structure-function analysis of the E2 proteins, in collaboration with Lubomir Turek. Trans-repression activity was mapped to the same domain as sequence specific DNA binding activity, suggesting that the E2 repressors may function by competitively inhibiting the binding of the E2 trans-activator to its recognition sequence, which we have previously shown to constitute an E2 dependent enhancer. This C-terminal domain, common to all three proteins, was also shown to mediate E2 dimer formation, raising the possibility that the E2 repressors may also inactivate the E2 trans-activator through the formation of

heterodimers. Sequence specific E2 trans-activation required that the N-terminal domain of the ORF be linked to the DNA binding domain by a peptide that could be varied in length and sequence. By itself, the N-terminal domain acted as a general trans-activator in that it activated, to a limited extent, all promoters tested, even though this domain had no detectable DNA binding activity. These results suggest that the mechanism of E2 trans-activation involves protein-protein interactions between a common transcriptional factor and the N-terminal domain of E2.

4. **BPV E1.** The E1 ORF of BPV encodes two trans-acting functions that are involved in the extrachromosomal replication of the viral genome. In collaboration with Herbert Pfister, we have determined that mutants mapping in both E1 functional domains have higher transforming activity than the wild type genome. These E1 mutant genomes were, as expected, integrated into the cellular DNA. Unexpectedly, their transcription rate in transformed cells was on average ten fold greater than the rate of transcription of the wild type genome. These results suggest either that integration of the BPV genome makes it unresponsive to a cellular repressor of viral transcription or that a full-length E1 gene product is a repressor of BPV transcription.

5. **Immortalization by BPV.** Since previous analyses of the biological activity of BPV had been conducted with permanent rodent cell lines, we decided to examine, in collaboration with François Cuzin, the immortalizing activity of the virus in primary rat embryo fibroblasts (REF). BPV was, by itself, able to induce immortalization of REFs. Genetic analysis of this activity revealed that the virus encodes two distinct classes of genes that cooperate in inducing immortality. When cotransfected with the neomycin resistant gene, E2 and E5 could independently induce resistant colonies but the colonies rapidly senesced. E6 alone could not induce colony formation, but when cotransfected with E2 or E5, it was able to induce immortalization of the resistant colonies. This was the first indication that E2 may have a direct effect on cell physiology. Consistent with this result, we have collaborated with Donald Young to demonstrate, using giant 2D gels, that E2 induces the expression of specific cellular proteins.

6. **HPV studies.** The HPV types commonly associated with cervical cancer, such as HPV16, have been shown by others to immortalize normal human keratinocytes, while those primarily associated with benign cervical lesions did not. It was therefore of interest to determine the specific HPV16 gene(s) that induce immortalization, since the activity of these genes could, in part, be responsible for the increased pathogenicity of HPV16. Our results demonstrated that E6 and E7 cooperate in the immortalization of normal neonatal foreskin keratinocytes. When transfected alone, E7 could induce transient proliferation but these cells eventually senesced. In contrast, E6 alone exhibited no activity, but when cotransfected with E7, the two genes induced cell lines that expressed both proteins and had indefinite growth potential. Changes in cellular gene expression are probably required for immortalization, since all of the keratinocyte lines examined were aneuploid. These results provide strong support for the hypothesis that the preferential maintenance and expression of E6 and E7 in human cervical carcinomas has pathogenic significance. In immortalized mouse NIH 3T3 cells, HPV16 DNA was found to induce anchorage independent growth. Mutational analysis, in collaboration with Dr. J. DiPaolo, indicated that E7 was the gene primarily responsible for this activity.

Given the biological activity of the E6 and E7, we thought it important to begin a biochemical characterization of the encoded proteins. We have determined that the E6 and E7 of HPV18 and BPV are zinc binding proteins. Since binding was observed only under reducing conditions, it is likely that binding is mediated by the cys-x-x-cys motifs that are conserved in both proteins. Similar motifs are characteristic of a class of sequence specific DNA binding proteins, and we have found that E6 and E7 have some avidity for DNA. However, unlike E2, they did not show sequence



specific binding to their respective genomes. In the absence of specificity, the biological significance of this binding activity remains uncertain.

7. Clinical studies. Following a report that papillomavirus DNA can be found in the smoke plume of warts treated by laser, we have, in collaboration with Paul Weber and Leonard Dzubow, studied a group of human and bovine warts to define further this potential risk of wart therapy for the operator. 5/8 vapors from human plantar warts treated by laser and 4/7 treated by electrocoagulation were positive for HPV DNA. Bioassay readily detected infectious BPV in vapor from bovine warts treated with either modality. More virus and viral DNA was usually found in laser-derived material. A surgical mask was capable of removing virtually all laser- or electrocoagulation-derived virus, strongly suggesting that such masks can protect operators from potential inhalation exposure to papillomavirus, and probably to other infectious agents.

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Lowy, D.R., and Schiller, J.T. Transforming genes of bovine and human papillomaviruses. In Concepts in Viral Pathogenesis, Vol. III, edited by Notkins, A.L., and Oldstone, M.B.A., Springer-Verlag, New York, in press.

Lowy, D.R. Clinical perspective - Papillomavirus infection. In Cancer Cells 7: Molecular Diagnostics of Human Cancer, edited by Furth, M., and Greaves M., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, in press.

Schiller, J., Vousden, K., Hawley-Nelson, P., Barbosa, M. Edmonds, C., Androphy, E., and Lowy, D. Analysis of Papillomavirus E6 and E7 gene products. In Papillomaviruses: A UCLA Symposium, edited by Howley, P.M., and Broker, T.R., Alan R. Liss, Inc., New York, in press.

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Hawley-Nelson, P., Vousden, K.H., Hubbert, N.L., Lowy, D.R., and Schiller, J.T.: HPV16 E6 and E7 proteins cooperate to immortalize normal human foreskin keratinocytes. Submitted.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09051-04 LCO

## PERIOD COVERED

October 1, 1988 through September 30, 1989

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

Biology of fps/fes oncogenes

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

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

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Oncology

## SECTION

## INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.00

## PROFESSIONAL:

1.00

## OTHER:

0.00

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

B

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

This project aims to elucidate the biological function(s) of the human c-fps/fes proto-oncogene and to understand the molecular basis of its oncogenic potential. Using efficient retroviral vectors, we showed for the first time that when normal human c-fps/fes is expressed at sufficiently high levels, it can cause tumorigenic transformation in mouse cells. In addition to level of expression, the transforming activity of c-fps/fes can be modulated at a second level, by incubation of cells overexpressing the c-fps/fes product NCP92 with low concentrations of a phosphatase inhibitor (sodium vanadate). This treatment potentiated the transforming activity of c-fps/fes by two orders of magnitude in a reversible, dose-dependent, and specific manner. Biochemical analysis indicated that the potentiating effect of vanadate was not secondary to an increase in the specific activity of NCP92 kinase but that it resulted from the stabilization of phosphotyrosine in the critical targets of NCP92. Further analysis indicated that NCP92 has a single tyrosine phosphorylation site, and that the phosphorylation of this site correlates with a potentiation of biological activity and an increase in the specific activity of NCP92 kinase. The potentiating effect of vanadate was found to be specific for the c-fps/fes pathway of transformation, providing functional evidence for the existence of distinct pathways of transformation for different tyrosine kinases.

### Major Findings:

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

We have previously shown that the expression of c-fps/fes at 5-10 times higher levels than endogenous levels in myeloid cells does not lead to cell transformation in NIH 3T3 transfectants. However, modification of the N-terminus of NCP92 by fusion with viral gag sequences, activated its oncogenic potential [Feldman et al., Oncogene Res. 1: 441-458 (1987)]. Although spontaneous chromosomal rearrangements involving the c-fps/fes locus have not yet been reported, this observation suggests that human c-fps/fes might also be susceptible to oncogenic activation by chromosomal alterations that modify the N-terminus of NCP92.

We have recently developed a highly efficient retroviral vector containing a F-MuLV long terminal repeat (LTR). By cloning the c-fps/fes gene into this new vector, we were able to show that when c-fps/fes is expressed at sufficiently high levels, this gene can induce tumorigenic transformation in mouse cells. NIH 3T3 cells transfected or infected with this construction expressed up to 200 times higher levels of c-fps/fes than endogenous levels in myeloid cells, and they formed foci of transformed cells. By contrast, an isogenic construction containing the parental Mo-MuLV LTR induced 3-10 times lower levels of c-fps/fes expression and its transforming activity was two orders of magnitude lower compared to the F-MuLV recombinant. This indicated that the transforming activity of c-fps/fes was a function of the level of expression NCP92 kinase. This level of expression correlated with the level of phosphotyrosine in cellular proteins of c-fps/fes-expressing cells. The transforming activity of c-fps/fes was significantly lower than that of its gag-activated counterparts. This may reflect differences in enzymatic activity because the N-terminal modified forms of c-fps/fes exhibit a 4-fold increase in kinase specific activity, and they induce higher levels of phosphotyrosine in cellular proteins in vivo.

In addition to level of expression, the transforming activity of c-fps/fes could be modulated by incubating cells that overexpress NCP92 with low concentrations of sodium vanadate. Vanadate is a known inhibitor of tyrosine phosphatases, and therefore, this treatment might be expected to prevent the dephosphorylation of NCP92 and its targets in vivo. At 4  $\mu$ M, vanadate potentiated the transforming activity of c-fps/fes by two orders of magnitude. To determine the mechanism of potentiation by vanadate, we carried out a biochemical analysis of the system. We first determined that vanadate did not act by increasing the levels of expression of NCP92, but that this inhibitor increased the level of  $^{32}$ P incorporation into NCP92. Phosphoamino acid analysis showed that this increase took place exclusively on tyrosine residues. The in vivo levels of phosphoserine and phosphothreonine in NCP92 were not

affected by vanadate treatment. This indicated that in our in vivo system vanadate functioned as an inhibitor of tyrosine phosphatases. Phosphopeptide analysis showed that the increase in tyrosine phosphorylation took place at the NCP92 autophosphorylation site, tyrosine 713. This was the only site of tyrosine phosphorylation in the molecule, and the vanadate-induced phosphorylation of this site correlated with a potentiation of biological activity and an increase in the specific activity of NCP92 kinase. These data suggest that tyrosine 713 may act as a positive regulatory site, and there are no sites in the NCP92 molecule whose phosphorylation inhibits NCP92 kinase activity. This is consistent with DNA sequence data which shows that no negative regulation site equivalent to tyrosine 527 in p60<sup>c-src</sup> exists in NCP92. To explain the potentiating effect of vanadate we considered two possibilities: the first was that this effect was secondary to an increase in the specific activity of NCP92, and the second was that potentiation was the result of the stabilization of phosphotyrosine in the critical targets of NCP92. Quantitative analysis showed that the vanadate-induced phosphorylation of tyrosine 713 had a positive effect on NCP92 kinase, but at 4  $\mu$ M vanadate, the increase in specific activity was only marginal (less than 20 %). On the other hand, the same treatment caused a 300 % increase in the level of phosphotyrosine in cellular proteins, measured by western blot analysis using anti-phosphotyrosine antibodies. This suggested that the potentiating effect of vanadate was not simply the result of a direct effect on the enzymatic activity of NCP92, but it was mostly due to the stabilization of phosphate in the critical targets of NCP92. Vanadate was able to discriminate between different fps/fes genes according to their relative transforming activity: its potentiating effect was maximum with c-fps/fes, it was nil with the highly transforming gag-v-fps/fes gene, and it was intermediate with the partially activated gag-c-fps/fes gene. The most likely explanation for this is that while the fully transforming v-fps/fes protein already phosphorylates its critical targets to an extent that is sufficient to transform most of the cells that are infected, the phosphorylating activity of NCP92 may not be sufficient to push all the cells that are infected to transformation. This illustrates the utility of a conditional system of transformation by c-fps/fes, where transforming activity can be modulated in response to different agents.

The most unexpected result we found was that even though vanadate is a general inhibitor of tyrosine phosphatases, its potentiating effect appeared specific for the c-fps/fes pathway of transformation. By contrast with c-fps/fes, vanadate was slightly inhibitory of the transforming activity of c-src. In case of c-src<sup>he527</sup>, a mutant that is structurally analogous to NCP92 in that its negative tyrosine regulatory site has been lost, there was no potentiation, but only a slight increase in transforming activity of less than two-fold. In case of EGF receptor there was also a slight decrease in transforming activity. These results indicated that the biological consequences of stabilizing phosphate in cells that overexpress different tyrosine kinases was different for each kinase. This differential response to the stabilization of phosphate represents in vivo evidence that even closely related tyrosine kinases have different mechanisms of action and may use different pathways to transform cells.



Publications:

Feldman RA, Lowy, DR, Vass, WC, Velu, TE. Transforming potential of the human c-fps/fes proto-oncogene. Submitted.

Feldman, RA, Lowy, DR, Vass, WC. Potentiation of c-fps/fes transforming activity by a phosphatase inhibitor. Submitted.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER  
 Z01 CB 05550-20 LCO

PERIOD COVERED  
 October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
**Retrovirus replication and cellular oncogene expression**

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

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

COOPERATING UNITS (if any)

Laboratory of Tumor Cell Biology, NCI, Dr. H. G. Guo  
 National Taiwan University Hospital, Taipei, Taiwan, Dr. C. Y. Chuang  
 Laboratory of Mathematical Biology, NCI, Dr. K. L. Ting

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

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CHECK APPROPRIATE BOX(ES)

- (a) Human 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 polymerase chain reaction (PCR) has been applied to 6 DNA samples from seropositive Taiwanese subjects to detect HIV-1 proviral sequences. The results suggest that these 6 samples contain various numbers of HIV-1 proviral copies which exhibit heterogeneity in their *env* sequences and are divergent from those of prototype HIV-1. Sequence analysis of two DNA clones, obtained by PCR-amplification of DNA from an asymptomatic homosexual, revealed the presence of an HIV-1 *env*-related sequence of 81 nucleotides containing 3 substitutions that were flanked by about 400 bp nucleotides of unknown origin. This interesting finding suggests that the provirus may be defective and integrated in cellular sequence, possibly adjacent to human an endogenous retroviral sequence. The biological and clinical significance of this phenomenon needs further investigation.

Screening of a cDNA library for a possible oncogene associated with the spontaneous reticulum cell neoplasm (RCN) of SJL/J mice resulted in several cDNA clones which hybridized with the RNAs of RCN more intensely than with those of normal lymphoid tissues. These cDNA clones were sequenced, and a homology search revealed highly repetitive LTR sequences which were derived from endogenous murine retroviruses. The presence of a flanking sequence which is partially homologous to an IL-2-like lymphokine sequence suggests that overproduction of this putative lymphokine-like substance by RCN may participate in the pathogenesis of RCN in SJL/J mice.

## Major Findings:

1. Detection by polymerase chain reaction of HIV-1 DNA in homosexuals, hemophiliacs, and AIDS patients in Taiwan. Although positive HIV-1 antibody tests such as ELISA and Western blot constitute evidence for previous infection, they do not provide information concerning the current presence of provirus or its extent of genomic variation. HIV-infection is usually followed by a latent period of several years before the appearance of AIDS symptoms, and very few lymphoid cells, probably 1 in 10,000, actually carry the virus. It is often unfruitful and time-consuming to attempt virus isolation by typical co-cultivation procedures. The polymerase chain reaction (PCR), which is an *in vitro* method for the primer-directed enzymatic amplification of specific DNA sequences, has recently been applied to detect HIV nucleotide sequences in AIDS-related materials. We collected DNA samples from seropositive Taiwanese subjects to detect HIV-1 proviral sequences by PCR. Peripheral lymphoid cells from an AIDS patient, an asymptomatic homosexual male, and two hemophiliacs, as well as formalized spleen tissues from two autopsied AIDS patients, were tested. Following PCR-amplification of DNA, the presence of proviral sequence was detected by specific probe analysis of Southern blots. All 6 samples exhibited a positive reaction when primer pairs from *gag* region or gp41 domain of *env* were used. Primer pairs derived from a relatively conserved region of gp120 domain of *env* also functioned to produce amplified fragments for all samples. However, when primer pairs covering domains spanning gp120 through gp41 were used, only four samples gave a definite amplified band, and one yielded a faint band. Primer pairs derived from *nef* and 3' LTR regions gave negative results. The relative intensity of the hybridization signal for each sample of PCR-amplified *gag* region did not correlate exactly with the signal strength of the various PCR-amplified *env* regions from each corresponding sample. The results suggest that these 6 samples contain various copy numbers of HIV-1 proviral sequences which exhibit heterogeneity in their *env* sequences and are divergent from those of the prototype HIV-1 clone. The DNA fragments amplified by PCR using primer pairs TN1 (24-mer) and TN2 (25-mer) which cover the gp120 region between them, i.e. nucleotides #7010-7660, were cloned into a Bluescript vector, and sequenced from both T3 and T7 ends. It was found that 81 nucleotides adjacent to TN2 were homologous with the *env* sequence of most prototype HIV-1 clones, except for substitutions in 3 nucleotides, while the rest of sequenced nucleotides (144 nucleotides from T3 end and 217 nucleotides from T7 end) were partially homologous to various regions of prototype HIV-1 *env* sequences. Although the origin of these partially homologous sequences is unknown, it is possible that cellular sequences including human endogenous retroviral sequences might have been co-amplified by the PCR procedure, a phenomenon that has been observed by other workers (B. J. Poiesz, SUNY Health Science Center, NY, personal communication). Further work is necessary to clarify the origin of these sequences. It is also possible that the integrated HIV-1 DNA might represent a defective provirus whose biological and clinical significance might be interesting subject for further study.

## 2. Search for oncogene associated with the spontaneous reticulum cell neoplasm (RCN) in SJL/J mice.

As reported previously, a cDNA library was constructed from the polyadenylated RNA of RCN tissues. The lambda-ZAP phage library carrying cDNA inserts was screened with various probes, including *v-abl*, LTR, and a 9-19 kb *HindIII* fragment of RCN DNA previously found to be present in RCN but not in normal tissues and to hybridize with *v-abl* and LTR probes. For each of the three probes, at least 10 different clones that were positive by hybridization were selected for further study. The cDNA inserts were isolated from these clones and were tested for their differential ability to hybridize with probes derived from radio-labelled cDNAs reverse-transcribed from polyadenylated RNAs of either RCN or normal tissues of SJL/J mice. Several inserts were found to react with the radio-labelled RCN probe more intensely than with the normal tissue probe. When

these cDNA inserts were sequenced, they were found to have homology to an IL-2-like lymphokine as well as to endogenous murine retrovirus LTRs. These preliminary findings may suggest that insertion of an endogenous proviral LTR in the vicinity of lymphokine-like gene may have induced enhanced production of a putative lymphokine which could contribute to the tumorigenesis of RCN.

Attempts to induce tumors in SJL/J mice by injection of syngeneic, normal splenic lymphoid cells which had been transfected by electroporation with each of the Bluescript vectors carrying the selected cDNA inserts have been negative thus far. Further observation is in progress, and experimentation with constructs equipped with appropriate promotor and selectable marker are being considered.

#### Publications:

Chang, K. S. S., Wang, L., Gao, C. Variants of amphotropic type-C retrovirus isolated from cultures of Moloney- and Rauscher-MuLV-induced tumors. *International J Cancer* 1988; 41:756-761.

Chang, K. S. S., Wang, L., Gao, C., Alexander, S., Ting, R. C., Bodner, A., Log, T., Kuo, A. F., Strickland, P. Concomitant infection of HTLV-I and HIV-1: Prevalence of IgG and IgM antibodies in Washington, D. C. area. *Europ J Epidemiol* 1988;4:426-434.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
 Z01 CB 04834-13 LCO

PERIOD COVERED  
 October 1, 1988 through September 30, 1989

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

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

PI:	S. S. Yang	Research Chemist	LCO NCI
OTHER:	J. V. Taub	Biolab Technician	LCO NCI
	M. Farshid	Guest Researcher	LCO NCI

COOPERATING UNITS (if any)  
 Seoul National Univ. Coll. Medicine, Seoul, Korea, Dr. B.J. Park  
 Dept. Biochemistry, Purdue Univ., West Lafayette, IN, Dr. Ronald Somerville  
 American Red Cross, Reference Lab., Rockville, MD., Dr. C. Fang

LAB/BRANCH  
 Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION  
 National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	1.5

CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	
<input type="checkbox"/> (a1) Minors			B
<input type="checkbox"/> (a2) Interviews			

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

The thrust of this study is to elucidate the genetic mechanism of neoplastic transformation of normal tissues. The experimental system focuses on a 3.1 kb moderately cell-transforming human oncogene, hhcM, isolated from an African hepatocellular carcinoma cell line, Mahlavu. Several related genomic DNA clones have now been isolated from other human hepatomas. Recently we have also isolated four cDNA clones each from human hepatoma and fetal liver DNA libraries. The cell-transformation capability of hhcM on both NIH3T3 cells and buffalo rat liver cells has been studied and the tumorigenicity of the transformants has also been described.

Determination of the nucleotide sequence of hhcM has now been completed. The hhcM nucleotide sequence shares no significant homology with other known oncogenes. It predicts an uninterrupted open reading frame specifying a message for a polypeptide of 467 amino acids, ca 52 kD. This was supported by the observation of a 52-55 kD polypeptide obtained in hybrid-select cell-free translation studies. This ORF was used in a chimeric construct with the lacZ promoter sequence, and production of the 52 kD protein is now scaled up significantly. The chimeric protein is being used to generate antibodies to be employed in characterization of the protein in cultured cells as well as in clinical samples of human urine, serum, and liver. Since earlier deletion analysis suggested that interruption of the nucleotide sequence within this ORF abolished the cell transformation capability, the possible direct involvement of this 52 kD protein in cell transformation is being explored.

Major Findings:

1. The nucleotide sequence of hhcM. We have previously shown that a 3.1 kb human genomic DNA sequence, hhcM, isolated from an African hepatocellular carcinoma (Mahlavu) cell line and cloned in a neomycin resistance-SV40 (pNeo) vector (designated pNrpM-1), transformed both NIH3T3 and Buffalo rat liver (BRL-1) cells at an efficiency of 1000 transformed colonies/ug. Both BRL-1 and NIH3T3 transformants transmitted the hhcM sequence stably in secondary and tertiary transfections, showed anchorage independent growth in soft agar and tumorigenicity in Swiss nu/nu mice. In this on-going project, we have completed the nucleotide sequence of both strands of the hhcM 3.1 kb DNA. Eleven M13 overlapping subclones carrying inserts of hhc<sup>9</sup>M DNA fragments in both directions were constructed. Using Sanger dideoxy sequencing and Maxam-Gilbert sequencing methods, the nucleotide sequence was determined in both directions. No significant homology was seen with known oncogenes. However there was a stretch of ca 300 nucleotides that shared homology with many other genes. These 300 nucleotides however were not part of the major open reading frame (ORF) described below.

2. Existence of an ORF coding for a 52-55 kD protein and its expression. Nucleotide sequence analysis of hhcM revealed an uninterrupted ORF of 467 codons with the potential to specify a protein of about 52-53 kD. This ORF resides at the 5' half of the hhcM sequence. It is preceded by the appropriate ribosomal RNA recognition nucleotide sequence and a TATAA box. The eucaryotic RNA polymerase II enhancer sequence (ATGCAAAT) was within 30 nucleotides of the start codon (ATG). A cell-free translation study was carried out to confirm this prediction. Total poly A rich RNA i.e. mRNA, was purified from log-phase Mahlavu hepatocellular carcinoma cells, using oligo-dT cellulose affinity chromatography. The hhcM specific mRNA was selected by hybridization of the poly A rich RNA with denatured hhcM 3.1 kb DNA, impregnated on a nitrocellulose filter. The unbound RNA was removed by appropriate washings and the hybridized hhc<sup>9</sup>M specific mRNA was released by heating in low salt buffer. The hhcM specific mRNA was then used to direct cell-free protein synthesis in a rabbit reticulocyte lysate assay with radiolabeled methionine. A few protein bands were identified as encoded by the hhcM specific mRNA, with strongest being a 52-55 kD protein.

The existence of this ORF coding for a 52 kD polypeptide was further confirmed by placing the appropriate sequence in an expression vector. The 52 kD expressing clone was constructed by removing 18 codons from the 5' terminus of the hhcM sequence via a Hinc II site and inserting a lacZ sequence, containing transcriptional and translational signals plus codons for 11 amino acids upstream. Kinetic analysis of the proteins produced by this plasmid carrying the chimeric construct showed the increasing synthesis of the 52 kD protein with time, and that the production level of hhcM protein was maintained at a high level for 24 hrs. A negative control construct, in which the lacZ-hhcM chimeric gene was interrupted by a Kanamycin cassette, failed to produce the 52 kD protein but synthesized instead the 28 kD aminoglycoside phosphotransferase protein. Currently we are developing a large-scale bacterial production of the 52 kD protein to immunize rabbits for antibody production.

3. Future use of anti-hhcM 52 kD antibody (anti-p52). One foreseeable application of this research is to obtain an antibody against the hhc<sup>9</sup>M 52 kD protein and use this antibody to study cross-reactivity with urine samples, blood serum, and liver biopsy samples from normal controls (young versus old), patients with (1) hepatocellular carcinoma, (2) liver infections (hepatitis viruses, A, B or delta), (3) liver cirrhosis from other abuses, e.g. alcoholism, aflatoxin and other microtoxins etc. and (4) hepatomegaly. The rationale for this resides on the fact that hhcM was isolated from Mahlavu hepatocellular carcinoma DNA, which showed 4 copies of intergrated but incomplete HBV sequences (Publication 1). Sera and urine samples were/will be obtained from the American Red

Cross Reference Lab. for Infectious Diseases (Dr. Chyang Fang, Director) or the NCI serum bank. Biopsy/autopsy materials include materials from Singapore and Hong Kong. They were/will be provided by Dr. G. Qian, Shanghai Cancer Institute, Dr. Robert C. Ting, Dr. B.-J. Park, Seoul National University Medical School, Korea, Dr. Chang (Korea), and Dr. B. Baroudy (NIAID), Gamble Research Institute, Cincinnati, Ohio. It is our aim to screen serum or urine protein by Western blot analysis with a radiolabelled anti-p52 antibody. Results from this screening study will enable us to assess the validity of anti-p52 for monitoring liver abnormality or viral infection with sera or urine. Furthermore, in situ hybridization with radio-labeled hccM DNA probe and/or radioimmunoassay with anti-p52 on frozen sections of biopsy/autopsy samples will be carried out (Dr. Baroudy) in order to determine the diagnostic value of anti-p52 in liver diseases and hepatocellular carcinoma.

4. Isolation of cDNA clones from human liver DNA libraries prepared from other hepatoma and from fetal liver. Using radio-labeled 3.1 kb hccM DNA as a probe, four cDNA clones were isolated from a human hepatoma cDNA library and four from a fetal liver cDNA library. Two of the hepatoma cDNA library clones, 35A bearing a single 1.6 kb insert and 34A bearing a long 1.7 kb insert and a short 0.3 kb insert, have been sequenced and compared with the hccM 3.1 DNA sequence. Clone 35A shared 70% homology with hccM but showed some rearrangements (Manuscript 4). The 1.7 kb cDNA sequence is an especially specific probe, as it lacks repetitive sequences and therefore has a low background of non-specific cross-reactivity. The expression of these clones will also be studied.

5. Histopathology of explants from tumors arising in NIH Swiss nu/nu mice challenged with pNrpM-1 transfected BRL-1 cells. Earlier observation of secondary tumor cells derived from an explanted hccM DNA transformed BRL-1 (#47) tumor had yielded a confusing morphology of cells with a fusiform appearance resembling fibroblast morphology. By monitoring microscopically tissue culture of the primary transformed BRL-1 (#47) tumor over a long period, we found that the epithelioid tumor cells changed morphologically to fibroblastic characteristic. This was especially prominent when the tumor cell culture was allowed to grow to high density. The histology of other primary tumors of hccM DNA transformed BRL-1 cells was clearly epithelioid and karyotype analysis confirmed their rat origin.

#### Publications:

Yang, S.S., Modali, R., Parks, J-B. and Taub, J. Transforming DNA sequences of human hepatocellular carcinomas, their distribution and relationship with hepatitis B virus sequence in human hepatomas, *Leukemia* 1988; 2: 102s-113s.

Yang, S.S., Vieira, W., Farshid, M, Cannon, G., and Wivel, N.A. Induction of B-Cell Lymphomas in Athymic NIH Swiss nu/nu Mice, *Leukemia* 1988; 2: 114s-124s.

Yang, S.S., Zhang, K., Yang, G.C. and Taub, J. Activation and specificity of aflatoxin B1 binding on human proto-oncogene and oncogene and cell transformation. In Pohland, A.E. and Richard, J. ed., *Cellular and Molecular Mode of Action of Selected Microbial Toxins in Food and Feeds*. Plenum Press, New York, N.Y. In press.

Yang, S.S., Zhang, K., Taub, J., Vieira, W. and Somerville, R. Complete nucleotide sequence of a 3.1 kb transforming DNA, hccM, isolated from Mahlavu hepatocellular carcinoma cells, and synthesis of a 52-kD and a 55-57 kD proteins encoded by hccM. In preparation.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08905-08 LCO (Formerly OD)
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Role of protein kinases in modulating cell growth and malignant transformation		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	W. B. Anderson	Research Chemist LCO NCI
OTHER:	Z. Kiss	Research Associate LCO NCI
	Z. Olah	Visiting Fellow LCO NCI
	C. Liapi	Visiting Fellow LCO NCI
	S. A. Lee	Biotechnology Fellow LCO NCI
	J. W. Karaszkiewicz	Biotechnology Fellow LCO NCI
	D. Lazega	Guest Researcher LCO NCI
	J. A. Strasburger	Technician LCO NCI
COOPERATING UNITS (if any) LVC, NCI, FCRF, Dr. U. Rapp Dept. Pathology, UCLA, Los Angeles, CA, Dr. R. Gopalakrishna Lab. Physiopath. Develop., Pathis, France, Drs. D. Evain-Brion, F. Raynaud		
LAB/BRANCH Laboratory of Cellular Oncology		
SECTION		
INSTITUTE AND LOCATION National Cancer Institute, Bethesda, MD 20892		
TOTAL MAN-YEARS: 6.8	PROFESSIONAL: 5.8	OTHER: 1.0
CHECK APPROPRIATE BOXES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The focus of this project is to better elucidate the possible involvement of transmembrane signal transmission systems in the regulation of cell growth and as possible targets in the process of tumor initiation and tumor promotion. Studies carried out to better elucidate the regulatory properties of protein kinase C (PKC) have established that this enzyme is markedly influenced by oxidative modification. Under mild conditions, treatment of the isolated enzyme or of intact cells with agents which generate reactive oxygen species leads to direct conversion of PKC to an activated form which is no longer dependent upon lipids. These results suggest that the interrelationship between the generation of oxygen free radicals and modulation of PKC may play a significant role in the mechanism of chemical carcinogenesis and tumor promotion.</p> <p>Results of immunohistochemical studies indicate that treatment of serum-deprived NIH 3T3 cells with the mitogen PDGF or with the PKC activator phorbol ester tumor promoter causes rapid activation of Raf-1 protein kinase at the nucleus. The data suggest that a cascade may be involved in the transmission of mitogenic signal from the plasma membrane through PKC to activation of the Raf kinase at the nucleus.</p> <p>Another line of investigation is to determine if altered phospholipid metabolism might be a primary event in the malignant transformation of cells. As with <i>ras</i> transformed cells, cells transformed with the <i>raf</i> oncogene were found to have significantly elevated levels of diacylglycerol. Results indicate that increased turnover of phosphatidylethanolamine (PE) may be, at least in part, responsible for the increased production of diacylglycerol in <i>raf</i> transformed cells.</p>		



### Major Findings:

1. Regulatory properties of protein kinase C. Protein kinase C (PKC) plays a crucial role in the transduction of transmembrane signals generated in response to a variety of hormones and other extracellular stimuli, and also serves as the intracellular receptor for phorbol ester tumor promoter (TPA). In the course of collaborative studies with Dr. R. Gopalakrishna to better understand the regulatory properties of PKC, we established that this enzyme is markedly influenced by oxidative modification. A selective modification of the regulatory domain of isolated PKC was obtained under mild oxidative conditions by protecting the catalytic site with ATP-Mg. Under these conditions there is a loss of both TPA binding and phospholipid-stimulated kinase activity. However, this modified form of the enzyme exhibits an increase in phospholipid independent activity. This suggests that selective oxidative modification of the regulatory domain may negate the requirement for Ca(2+) and lipids for activation. The treatment of intact C6 glioma or B16 melanoma cells with hydrogen peroxide also resulted in the generation of an oxidatively modified isoform of PKC that exhibits activity in the absence of phospholipids. These results may relate to the mechanism of tumor promotion by agents such as benzoyl peroxide and cumene hydroperoxide which do not bind directly to PKC, but nonetheless appear to activate PKC by enhancing reactive oxygen species within the cell.

2. Raf protein kinase. Since PKC plays a major role in cell growth regulation and tumor promotion, studies have been aimed at identifying proteins and activities modified by PKC to in turn transduce a signal to other components of the cell, including the nucleus. One activity found to be modified by initial activation of PKC and which may play an important role in the signal transduction cascade is the serine/threonine protein kinase encoded by the raf proto-oncogene. Studies, in collaboration with Dr. U. Rapp, suggest that Raf protein kinase may be directly activated by PKC. Further, we have observed that treatment of growth arrested NIH 3T3 cells with the mitogen PDGF (maximal at 5 min.) or with phorbol ester tumor promoter (maximal at 20 min.) provokes a rapid redistribution (along with activation) of the Raf protein kinase to the perinuclear region.

Since little is known concerning the biochemistry of either cellular Raf kinase or the truncated *v-raf* protein kinase product of the *v-raf* oncogene, studies were initiated to isolate and characterize these protein kinase activities. An *in vitro* phosphorylation assay was developed utilizing a synthetic pseudosubstrate peptide as phosphate acceptor, and partial purification was attained by FPLC. These studies should lead to a better understanding of the cascade of events leading from initial generation of a signal at the plasma membrane to transduction of the signal to the nucleus.

3. Altered phospholipid metabolism. The modulation of phospholipid metabolism and generation of the second messenger diacylglycerol (DAG) is thought to play a primary role in initiating cell proliferation in response to certain growth factors, and also is thought to be a potential target of certain oncogene products which act at the cell membrane. We have found a 3-fold increase in the intracellular level of DAG when NIH 3T3 cells are transformed by human or mouse A-raf or 208 F rat fibroblasts are transformed by an activated (truncated) mouse c-raf. With membranes isolated from ethanolamine-prelabeled *raf*-transformed cells, 75% more phosphatidylethanolamine (PE) was hydrolyzed during a 40 minute incubation period compared to membranes from untransformed cells. A similar high rate of PE turnover also was observed with intact raf transformed cells. Analysis of water-soluble degradative products of PE suggest that a phospholipase D-type enzyme is involved in the increased turnover noted in the *raf*-transformed cells. Since little change is noted in the turnover of either phosphatidylcholine or phosphatidylinositol, the data suggest that phospholipase D-mediated turnover of PE contributes to the elevated levels of DAG found in raf-transformed cells. In studies to characterize the phospholipase D-mediated hydrolysis of PE, it was found that TPA

treatment increased this activity. However, transformation of NIH 3T3 cells with *raf* was observed to negate the ability of TPA to enhance sphingomyelin synthesis. These results further suggest a possible interrelationship between PKC and raf kinase to regulate biological processes.

4. Retinoids in mediating cell growth and tumor promotion. The intent of this study is to elucidate the role and mechanism of action of retinoids in mediating cell growth and differentiation, and in acting as antagonists toward tumor promotion. Previously, we established that treatment of cells with retinoic acid (RA) leads to increased cyclic AMP-dependent protein kinase (PKA) activity, suggesting an important synergy between RA and cyclic AMP to regulate cell growth and differentiation. Now we observe that treatment of PYS (parietal endoderm) cells with TPA causes a rapid (within 10 min.) increase in cytosolic PKA activity. Of interest is the finding that the anti-tumor promoter RA completely abolished the TPA effect on PKA, even though it had no effect on initial TPA activation of PKC.

In collaboration with Dr. D. Evain-Brion, studies were continued to better understand the role of decreased PKA in the hyperproliferative skin disease psoriasis, and to further study the mechanism by which RA treatment of psoriatic cells increases their low PKA levels toward normal. In a study involving 34 psoriatic patients, it was found that the level of the RI regulatory subunit of PKA is significantly decreased in the membranes of red blood cells obtained from psoriatic subjects when compared to the level of RI found in red cell membranes of normal subjects. A significant negative correlation is noted between the level of RI found in the red cell membranes and the severity of the disease in the psoriatic patients. Further, it was found that oral retinoid treatment of psoriatic patients increased RI levels toward normal, thus paralleling results previously obtained with *in vitro* RA treatment of psoriatic fibroblasts. Together with the TPA effects, these results suggest that changes in PKA activity may be involved in the antineoplastic and therapeutic actions of retinoids.

#### Publications:

Plet, A., Gerbaud, P., Anderson, W.B., and Evain-Brion, D: Effect of phorbol ester on cAMP-dependent protein kinase in PYS teratocarcinoma cells with retinoic acid. *Cancer Res* 1988; 48: 3993-3997.

Ho, A.K., Thomas, T.P., Chik, C.L., Anderson, W.B., and Klein, D.C. Protein kinase C: Subcellular redistribution by increased Ca (2+) influx. *J Biol Chem* 1988; 263: 9292-9297.

Kiss, Z., Rapp, U.R., and Anderson, W.B. Phorbol ester stimulates the synthesis of sphingomyelin in NIH 3T3 cells: A diminished response in cells transformed with human A-raf carrying retrovirus. *FEBS Lett* 1988; 240: 221-226.

Raynaud, F., Liapi, C., Gerbaud, P., Anderson, W.B., and Evain-Brion, D. Effect of retinoic acid on cAMP-dependent protein phosphorylation in psoriatic fibroblasts. *Biochem. Biophys Res Commun* 1988; 156: 263-268.

Rossi, M.A., Anderson, W.B., and Dianzani, M.U. Effect of 4-hydroxynonenal on PIP2 phospholipase C activity modulated by GTP. *Proceedings of the 19th Congress of the Italian Society of Pathology* 1988; pp 49-52.

Kiss, Z. and Anderson, W.B. Phorbol ester stimulates the hydrolysis of phosphatidylethanolamine in leukemic HL-60, NIH 3T3, and baby hamster kidney cells. *J Biol Chem* 1989; 264: 1483-1487.

Rapp, U.R., Heidecker, G., Huleihel, M., Cleveland, J.L., Choi, W.C., Pawson, T., Ihle, J.N., and Anderson, W.B. raf Family Serine/Threonine protein kinases in mitogen signal transduction. Cold Spring Harbor Symposia on Quantitative Biology, 1989; Vol 53: pp. 173-184.

Gopalakrishna, R., and Anderson, W.B. Ca (2+) and phospholipid-independent activation of protein kinase C by selective oxidative modification of regulatory domain: Proc Natl Acad Sci USA 1989; in press.

Raynaud, F., Gerbaud, P., Enjolras, O., Gorin, I., Donnadieu, M., Anderson, W.B., and Evain-Brion, D. Decreased cAMP binding to the RI regulatory subunit of cAMP-dependent protein kinase in erythrocyte membranes of patients with psoriasis. Lancet 1989; in press.

SUMMARY REPORT  
LABORATORY OF PATHOLOGY  
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS  
NATIONAL CANCER INSTITUTE  
October 1, 1988 to September 30, 1989

The Laboratory of Pathology is responsible for all the diagnostic services in anatomic pathology for the Clinical Center of the NIH and has research programs in various areas of experimental pathology. A fully accredited 4-year residency program in anatomic pathology is provided for 9 residents and 3 fellows. The Laboratory is divided into 9 sections:

Surgical Pathology Section (Dr. Maria J. Merino, Chief)  
Pulmonary and Postmortem Section (Dr. William D. Travis, Chief)  
Cytopathology Section (Dr. Diane Solomon, Chief)  
Ultrastructural Pathology Section (Dr. Maria Tsokos, Chief)  
Biochemical Pathology Section (Dr. David D. Roberts, Chief)  
Tumor Invasion and Metastases Section (Dr. Lance A. Liotta, Chief)  
Hematopathology Section (Dr. Elaine S. Jaffe, Chief)  
Gene Regulation Section (Dr. David L. Levens, Chief)  
Office of the Chief (Dr. Lance A. Liotta, Chief)

All sections conduct investigative work and provide research opportunities for the residents. Investigative work completed or in progress is listed by section as follows.

Surgical Pathology Section

Approximately 6,000 surgical specimens or biopsies (approximately 60,000 slides) were accessioned in the past year. Approximately 1,000 specimens of fresh human tissues, including the eyes which are regularly removed during a complete autopsy, were furnished to NIH scientists in various laboratories. A diagnostic immunohistology service is provided for clinical diagnosis and research. A tissue procurement nurse works closely with the staff and helps in the distribution of specimens to scientists. Clinicopathological studies are provided supporting clinical protocols conducted by the Surgery Branch, the Medicine Branch, and the Clinical Center.

Dr. Merino is investigating the use of immunohistochemical markers to study the growth fraction and marker heterogeneity of human breast carcinomas over a range of histologic types, before and after therapy. The goal will be to correlate the marker levels with relapse rates and response to therapy.

Pulmonary and Postmortem Section

Dr. Travis is conducting a detailed review of interstitial fibrotic lung disease. Lung biopsies from patients with idiopathic pulmonary fibrosis have been reviewed and the data are currently being analyzed. Biopsies from 30 patients with pulmonary histiocytosis X are also being studied. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings.



The pulmonary and autopsy pathological material is being actively utilized by the staff and residents for research projects involving clinicopathological correlation and pathological characterization of diseases studied at the Clinical Center. Electron microscopic and immunocytochemical techniques are being applied to the study of these diseases. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH.

The neuropathology service is integrated with the Surgical Pathology Section, Pulmonary and Postmortem Section, and the Ultrastructural Pathology Section. Neuropathology diagnostic (patient care) service and teaching (of pathology residents) are provided. Approximately 250 neurosurgical specimens were examined last year. The service also functions in a collaborative manner to provide neuropathological support for a wide range of clinicopathologic investigations, including dementia, pituitary adenomas, PML and gliomas.

#### Cytopathology Section

This section provides diagnostic services in cytology (both exfoliative and fine needle aspiration). During the year 3800 cytology specimens were accessioned. Special expertise is provided in aspiration cytology. Fine needle aspirations have increased 100%. The section provides a) a pathologist to perform the aspiration; b) a pathologist to instruct the clinician in aspiration technique; c) a cytotechnologist to assist the clinician; d) assistance to the radiologist during the aspiration of a deep tissue specimen.

The section organized three national meetings to address quality assurance in cytology and to develop a new system for reporting cervical/vaginal cytopathology diagnoses.

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 applied immunocytochemistry to the diagnosis of lymphomas in cytological materials. One hundred and ninety-eight tumor suspensions have been evaluated for tumor-associated antigens and for phenotypic analysis of tumor infiltrating lymphocytes.

#### Ultrastructural Pathology Section

This section provides diagnostic electron microscopy services for a diverse group of Clinical Center physicians, including NCI, NIAMDD, NHLBI, NIAID, and NINCDS, as well as submitted cases from outside physicians. This past year approximately 200 cases were accessioned; over 150 were processed and diagnosed. This facility provides diagnostic training and clinical research opportunities for residents and fellows. Dr. Maria Tsokos has been doing diagnostic EM while investigating the factors such as expression of MyoD1 gene products which correlate with the biologic aggressiveness of alveolar rhabdomyosarcoma compared to embryonal rhabdomyosarcoma.

P-glycoprotein has been associated with a multidrug-resistant (MDR) phenotype in a wide variety of animal and human tumor cell lines and clinical tumor specimens. Pediatric tumors, however, have not been included in the studies reported so far. Tissues from Ewing's sarcomas, primitive neuroectodermal tumors and rhabdomyosarcomas will be evaluated for the presence of P-glycoprotein using a commercially available antibody against P-glycoprotein and employing a modified immunoperoxidase "sandwich" staining method. Hyperdiploidy has been associated with an unfavorable histology (anaplastic) in Wilms' tumor and with a better response to chemotherapy in unresectable neuroblastoma. DNA-aneuploidy was found in rhabdomyosarcoma (RMS) in contrast to Ewing's sarcoma and primitive neuroectodermal tumor (PNET).

The stage of differentiation of the 6 rhabdomyosarcoma (RMS) cell lines will be altered by agents and/or conditions previously used to induce or inhibit differentiation in myoblastic cell cultures and RMS cell lines.

#### Biochemical Pathology Section

The Biochemical Pathology Section, under Dr. David Roberts, is carrying on research on immunochemistry of complex carbohydrates and cell surface receptors. 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.

The functions of thrombospondin in tumor cell adhesion and migration are being investigated. The carboxyl-terminal domain mediates attachment and haptotaxis, while the amino-terminal domain mediates cell spreading and chemotaxis. One class of receptors are sulfated glycoconjugates which bind to the amino-terminal domain of thrombospondin. A minor heparan sulfate proteoglycan that binds thrombospondin with high affinity was identified in two melanoma cell lines and purified by affinity chromatography on thrombospondin-Sepharose. The intracellular responses of cells to surface receptor binding of thrombospondin to the two types of receptors are also being investigated.

Sulfated glycolipids and  $\alpha$ 2-3-linked sialyl glycoproteins are two independent receptors for cytoadhesion of M. pneumoniae. The former is present at high concentrations in human trachea which is the primary site of infection. Monoclonal antibodies to the pathogen will be used to define receptors for these, and the respective carbohydrates on affinity matrices will be used to purify their receptors.

Several serotypes of Chlamydia were examined for binding to glycolipids and glycoproteins. The elementary bodies bind specifically to glycolipids containing GalNAc $\beta$ 1-4Gal $\beta$ 1-4Glc sequences, heparin or fucoidin conjugated to albumin, and to the adhesive proteins fibronectin and laminin.

Dr. Roberts has determined that sulfated glycolipids participate in tumor cell adhesion by directly promoting adhesion and as receptors for thrombospondin on melanoma cells.

Glycolipids isolated from esophageal tissues obtained from cancer patients, normal individual controls, and a cultured esophageal carcinoma line were examined using several monoclonal antibodies. Both neutral and sialylated glycolipids containing 3-fucosyllactosamine sequences differ in expression between the tumor and control tissues and a heptaglycosylceramide reacting with antibodies to this sequence is found only in the tumor tissues. These glycolipid antigens may be useful as markers of esophageal carcinoma.

#### Tumor Invasion and Metastases Section

Tumor invasion and metastasis is the major cause of cancer treatment failure. Up to 60% of patients with newly diagnosed solid tumors already have microscopic or clinical occult metastasis. Even if the primary tumor can be eradicated, the metastatic disease will progress and kill the patient. Individual patients' tumors vary widely in aggressiveness. Some tumors grow to a large size and fail to metastasize. In contrast, some tumors disseminate very early in their development. Thus, there is a great clinical need to develop new methods to a) predict the aggressiveness of an individual patient's tumors, b) identify clinically occult micrometastasis, and c) eradicate established metastatic colonies. In order to provide a rational basis for clinical strategies, our laboratory has been studying gene products involved in tumor invasion and metastases.

The TIM section members have identified a set of gene products which may regulate tumor invasion and metastasis: a) laminin receptor; b) autocrine factor; c) NM23 metastasis suppressor protein, d) type IV collagenase, and e) CSC-21 metalloproteinase inhibitor. These metastasis-associated gene products may be different from those which regulate tumor growth, and this is in keeping with the concept that benign tumors may grow in an unrestrained fashion, but fail to invade.

The laminin receptor is a cell surface protein which binds laminin, a glycoprotein of basement membranes, and is augmented in certain aggressive human cancers compared to non-malignant counterpart cells. Dr. Mark Sobel in our section has cloned and sequenced the full-length cDNA for the laminin receptor. Dr. Castronovo, a fellow in our section, has recently found that estrogen and progesterone regulate expression of the laminin receptor gene. Using Northern blotting, estrogen and progesterone treatment increased laminin receptor mRNA ten fold in ER and PgR positive breast cancer cell lines. In contrast, ER and PgR negative breast cancer cells exhibited a constant high level of laminin receptor mRNA unaltered by hormone treatment. These observations of augmented levels of cell surface laminin receptors in human tumors has led to the therapeutic strategy of using anti-laminin receptor antibodies to target adriamycin encapsulated liposomes to tumor cells. In vitro studies using this approach have demonstrated more than a ten-fold higher rate of killing of human breast carcinoma cells compared to breast epithelial cells. Clinical trials using this approach are in the preclinical toxicology phase.

The autocrine motility factor (AMF) is a new cytokine which is secreted by tumor cells, binds with high affinity to a cell surface receptor, and profoundly stimulates locomotion. Dr. Elliott Schiffmann's group has found



that a pertussis toxin sensitive G protein is required for the transduction of the AMF signal through the phosphoinositol pathway. Screening of inhibitors blocking this pathway has led to the identification of a Merck compound which may be of therapeutic value in cancer treatment. Preclinical toxicology and the design of clinical protocols are now complete for this compound. We hope that suitable clinical support will be available to immediately initiate clinical trials.

We have recently published a clinical study describing the measurement of AMF in the urine of patients with transitional cell carcinoma of the bladder. A very strong correlation was found between the AMF levels and tumor grade, stage of invasion, and recurrence. This test has received great interest by urologists and we are now accepting hundreds of urine samples for a large screening study. The test may be a potential adjunct in the early detection of bladder cancer recurrence.

NM23 is a gene expressed to a higher level in low metastatic tumor cells compared to high metastatic cells. It encodes a putative metastases suppressor protein. Dr. Pat Steeg, a fellow in our section, has cloned and sequenced a full-length NM23 cDNA clone. The encoded protein sequence has properties suggesting a leucine-zipper DNA binding protein. The NM23 protein has a size of 17 kDa by Western blotting of nuclear extracts using anti-peptide antibodies. The NM23 protein is virtually identical to a Drosophila gene (*awd*) product which plays a critical role in morphogenesis and tissue pattern formation. The discovery of NM23 therefore provides a link between development and metastasis. The negative correlation of NM23 expression with metastases was confirmed in four different animal model systems using multiple clones. Immunogenicity does not appear to play a role in the mechanism of action of NM23. A strong negative correlation was found between human breast carcinoma aggressiveness, in terms of lymph node metastases, and NM23 mRNA expression. Preliminary data suggests that the NM23 gene location may be on chromosome 11, possibly on the short arm region shown by Callahan to be deleted in more aggressive human breast cancer cases. Additional preliminary data has demonstrated a strong suppression of experimental metastases in a series of tumor cell clones transfected with NM23 compared to a series of control transfections. NM23 may provide a number of significant prognostic and therapeutic strategies.

The structural backbone of the basement membrane is type IV collagen, a collagen genetically distinct from the other types of collagen. We originally determined that type IV collagen was resistant to known mammalian collagenase which could degrade collagen types I, II, and III. Our laboratory discovered type IV collagenase, a metalloproteinase which cleaves type IV collagen in a pepsin resistant site located 1/4 of the distance from the amino terminus of the substrate. The enzyme is secreted in a latent 70 kDa form which fails to degrade type IV collagen. When the enzyme is activated, it becomes reduced to a molecular size of 62 kDa and can specifically cleave type IV collagen. Both latent and active forms of this enzyme are gelatinolytic. This enzyme is secreted in large amounts by tumor cells and its rate of elaboration is correlated with the metastatic phenotype in several systems. It is a unique member of the metalloproteinase family. Antibodies which block the activity of this enzyme and synthetic inhibitors of this enzyme block tumor cell



invasion in vitro. This enzyme may play a crucial role during invasion and metastasis.

Analysis of the full-length cDNA clone encoding the human latent type IV collagenase has revealed three separate functional domains: 1) an amino terminal domain of 80 residues which triggers a conformational change in the enzyme resulting in activation from a latent state. Organomercurial activation of the latent type IV collagenase results in autocatalytic removal of this amino terminal domain resulting in the conversion to a 62 kDa activated form of the enzyme. The amino terminal sequence of the purified enzyme before and after activation reveals cleavage at a single locus generating a new amino terminus starting at residue 81: YNFFPPKPKW DKNQ. This site is adjacent to the carboxyl end of a conserved region PRCGVPDV which contains an unpaired cystein residue. An identical sequence is present at the activation site of prostromelysin and interstitial procollagenase. Further studies have demonstrated that this sequence comprises an intrinsic inhibitor which blocks the active site of the enzyme. (2) a cystein-rich repeat domain which binds to the substrate independent of metal ions and is responsible for the substrate specificity, and 3) a histidine containing domain VAAHEFGHAMGLEHSQ which is the active site of the enzyme, and requires metal ions. Affinity purified antibodies made against synthetic peptides corresponding to the latent versus active enzyme were used to study the expression of the enzyme antigen during progression from human in situ to invasive breast carcinoma. The latent type IV collagenase was found associated to a low degree with benign duct myoepithelial cells. In contrast, the active enzyme form was not present in normal ducts or benign ductal proliferation, was present variably in comedo carcinoma in situ, and was markedly augmented in primary invasive carcinoma and metastases. This indicates that enzyme activation may be an important correlate of tumor progression. Type IV collagenase production also shows a strong correlation with invasive grade in human colorectal cancer, and is markedly augmented following ras oncogene transfection in a variety of murine and human cell types.

Identification of functional domains on type IV collagenase has led us to devise three strategies for inhibition of the enzyme using synthetic peptides: a) blocking activation of the latent metalloproteinase, b) blocking binding of the latent or active metalloproteinase to the substrate, and c) inhibition of the active (cleavage-producing) site of the enzyme. We have discovered specific synthetic peptides which accomplish all three strategies.

The first clinical application of this information is in the diagnosis and prognosis of cancer. Immunohistologic staining using antibodies to various domains on type IV collagenase have proven to be a useful means to judge the invasive potential of a tumor and to identify micrometastases in lymph nodes or other tissues distant from the primary tumor site. Type IV collagenase may be a particularly good marker in urine for bladder cancer, and may also prove useful as a serum marker for systemic cancers. Preliminary data indicates that levels of type IV procollagenase mRNA and type IV procollagenase gene rearrangement and amplification may be of prognostic value in human carcinomas.

The second clinical application is therapy. We believe that synthetic, natural, or recombinant inhibitors of type IV collagenases may have significant therapeutic potential as agents which block angiogenesis and tumor invasion. They may have low toxicity since basement membrane turnover is rare in normal tissues. There also may be therapeutic utility for the recombinant enzyme itself or for enzyme fragments.

In regard to the regulation of type IV collagenase, we have discovered a novel proteinase inhibitor which binds with a 1:1 stoichiometry to type IV collagenase. The inhibitor has been purified to homogeneity and the complete primary structure has been determined. The novel inhibitor, termed CSC-21, is the first new member of the TIMP (tissue inhibitor metalloproteinases) family with conservation of all 12 cysteine positions. This new inhibitor can be considered in the class of tumor suppressor gene products.

### Hematopathology Section

The Hematopathology Section conducts a major program in diagnostic and experimental hematopathology. Dr. Jaffe supervises an internationally recognized consultation service for diagnostic hematopathology. While most of this material pertains to patients with malignant lymphoma admitted to the clinical services of the National Cancer Institute, collaborations are also conducted with NIAID and NIAMDD for patients with reactive lymphoproliferative lesions. Drs. Jaffe and Cossman also receive several hundred cases submitted for diagnostic consultation from pathologists in the regional medical community as well as throughout the United States and other parts of the world.

The Hematopathology Section continues its active research program on the immunological characterization of malignant lymphomas. All patients with newly diagnosed lymphomas or recurrences are studied for phenotypic and functional markers. This information is utilized to study the relationship of malignant lymphomas to the normal immune system, to develop improved classifications of disease, and to distinguish new clinicopathologic entities. This information is also being used as a basis for immunotherapy in collaboration with the Medicine Branch, DCT, and the Biological Response Modifier Program in Frederick, Maryland, NCI.

Dr. Jaffe has continued to study the pathologic features of HTLV-I associated lymphomas. In selected populations where HTLV-I is endemic, such as Jamaica, prospective studies of all newly diagnosed lymphoma patients are conducted. Such studies are useful in identifying the clinicopathologic spectrum of HTLV-I associated diseases. Prospective studies of all lymphomas in similar geographic regions with differing incidences of adult T cell leukemia/lymphomas are studies to discern factors which make an impact on the incidence of HTLV-I and HTLV-I associated diseases.

Lymphocytes activated with IL2 both in vivo and in vitro are being used for the experimental treatment of human malignancies. The mechanism of action of this therapy is not clearly established, and it is not known whether those lymphokine-activated killer cells (LAK) infused actually migrate and infiltrate tumors, or whether other effector cells mediate the observed tumor regression.

In order to better understand the pathophysiology of LAK-IL2 treatment, tumor biopsy specimens are obtained before, during, and after conclusion of LAK-IL2 treatment. Specimens are studied with an immunohistochemical technique for a battery of tumor markers as well as markers capable of identifying T cells, B cells, histiocytes, NK cells, and other effector cells. These data are then correlated with conventional, clinical and pathologic data to determine whether the immunopathology observed can predict clinical response.

Dr. Cossman has been studying the molecular basis of the diagnosis of human lymphoproliferative disease. This work has already produced a number of major findings. The T- and B-cell lineage of diffuse, aggressive lymphomas as determined by phenotypic studies can be confirmed by detection of appropriately rearranged T-cell receptor or immunoglobulin genes. Moreover, in some patients' tissues which lack evidence of malignant lymphoma by conventional histology or immunologic studies, malignant lymphoma can be identified by these techniques.

Dr. Maryalice Stetler-Stevenson has demonstrated that frequent relapse of follicular lymphoma, the major obstacle to cure, is a consequence of clonal expansion of daughter cells derived from a common stem cell. Thus, despite the "clinical" remission achieved by therapy in most patients, residual lymphoma cells must persist. To detect occult lymphoma, she has specifically amplified the joined *bcl-2/JH* DNA sequences created by the *t(14;18)* translocation seen in nearly all follicular lymphomas. Using multiple rounds of primer-directed DNA polymerization (polymerase chain reaction, PCR), he can detect 1 copy of *bcl-2/JH*, which is four orders of magnitude more sensitive than flow cytometry or Southern blot restriction analysis. Genomic DNA sequence analysis of four lymphomas confirmed that the size of the amplified fragment serves as a unique tumor marker. Direct application to clinical samples has demonstrated lymphoma cells which were otherwise undetectable.

#### Gene Regulation Section

Dr. David Levens has developed a novel method for identifying sequence-specific DNA binding proteins.

The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. These same proteins also bind to a negative *cis*-element approximately 330 bp upstream of *c-myc* promoter P1. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not in itself possess full specificity. The second component does not in itself bind DNA, but upon forming a complex with the first component confers greatly enhanced power to discriminate between different sequences.

Application of an exonuclease assay developed in his laboratory to identify and map the sites of tight protein-DNA interactions on large pieces of DNA, to the c-myc has revealed multiple cis- and trans-elements both upstream and downstream of the c-myc promoter P1. The group has focused on 3 regions, each of which interacts with one or more sequence-specific binding proteins. A negative cis-element present in intron one of the c-myc gene is frequently mutated in Burkitt's lymphoma.

Office of the Chief

Dr. Mackem is investigating genes which regulate morphogenesis. The elucidation and characterization of genes necessary for establishing pattern formation during morphogenesis and the study of their regulation are problems which are central to many aspects of vertebrate biology. It may be expected that processes including responses to trophic stimuli, cell-cell interactions, migration, differential cell multiplication, programmed cell death, etc. are all involved. Many of these same processes are recapitulated in a pathologic manner during oncogenesis.

Dr. Davis is developing a system to study regulatory events in leukocyte adhesion and migration. HL-60 promyelocytic leukemia cells are known to be deficient in cell-substrate adhesive ability unless treated with differentiating agents which can induce either a granulocyte-like or monocyte-like phenotype. Dr. Davis has treated HL-60 cells with various known differentiating agents such as phorbol esters (TPA), dimethylsulfoxide (DMSO), 1,25 dihydroxyvitamin D3 (vitamin D3), dibutyryl cyclic AMP and retinoic acid and have examined the development of cell-substrate adhesive and migratory ability as well as the presence of proteolytic activities. The cells release a 94 kDa gelatin-degrading metalloprotease which is induced during the development of cell-substrate adhesive ability with phorbol esters.

Dr. Kelly is investigating the consequences of mitogen-mediated signals to T cells. She has isolated over 60 novel cDNA clones that represent mRNA species induced by PHA and PMA in human peripheral blood T cells. Primary sequence analyses on five clones have been completed and have revealed two functional classes of proteins encoded by these genes: lymphokines and DNA binding proteins/transcription factors. Potential functional activities of the three putative lymphokines currently are being tested with recombinant proteins.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00853-36 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Surgical Pathology

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

PI: M.J. Merino

Chief, Surgical Pathology Section

LP NCI

OTHER: (see next page)

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Surgical Pathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

D

## SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The Surgical Pathology Section provides service in anatomic pathology for the Clinical Center patients and collaborates with the research staff of all institutes in those investigations which involve the use and study of human pathological material. The frozen section and surgical pathology processing area was constructed adjacent to the new operating rooms and has been in use since April, 1983. This new facility has greatly enhanced processing of specimens and communication of diagnostic findings with attending physicians. It is equipped with intercom and television viewing screens in each operating room to facilitate communication.

The staff is actively engaged in a variety of projects involving clinicopathological correlation and pathologic characterization of diseases studied at the Clinical Center. Up-to-date immunohistochemical techniques have been applied to the study of tumors and other non-neoplastic diseases. The use of immunohistochemical staining has greatly facilitated more precise diagnosis in selected cases and with the increasing number of monoclonal antibodies available this technique should have even greater value in diagnostic and research pathology. A major renovation of the histology laboratory is in the final stage. This will include space allocated for performance of special stains and immunocytochemistry.

Other Professional Personnel:

P. Cohen	Senior Staff Fellow	LP NCI
W. Travis	Expert	LP NCI
M. Raffeld	Senior Staff Fellow	LP NCI
C. Axiotis	Expert	LP NCI
<sup>†</sup> S. Pittaluga	Visiting Associate	LP NCI
<sup>†</sup> G. Jaffe	Staff Fellow	LP NCI
<sup>†</sup> G. Davis	Staff Fellow	LP NCI
<sup>†</sup> K. Schmidt	Staff Fellow	LP NCI
<sup>†</sup> A. Ginsberg	Staff Fellow	LP NCI
<sup>†</sup> J. Taubenberger	Medical Staff Fellow	LP NCI
<sup>†</sup> D. Kleiner	Medical Staff Fellow	LP NCI
<sup>†</sup> D. Roth	Medical Staff Fellow	LP NCI
<sup>*</sup> J. Stern	Consultant in Dermatopathology	

Objectives:

The objectives of the Surgical Pathology Section are:

- (a) to provide diagnostic services in pathologic anatomy to the clinical research projects conducted at NIH;
- (b) to carry out independent research;
- (c) to provide a residency program in anatomic pathology; and
- (d) to collaborate with investigators in research involving the use and study of human materials.

The proposed course of research includes (a) continuing to provide the services described; (b) increasing the interaction with the clinical branches in the design and evaluation of protocols; (c) improving the opportunities for the resident staff to participate in teaching, conferences, and seminars, and providing elective periods to be spent accomplishing research projects with the senior staff; and (d) implementing data retrieval programs.

The staff assists the residents in preparing for the numerous clinical conferences in which the section participates.

Histopathologic and immunohistology studies will be performed as part of the following clinical protocols: 1) Dose intensive chemotherapy in locally advanced and metastatic breast cancer; 2) Use of monoclonal antibody L6 to scintigraphically detect metastases; 3) Combination radioiodine and adriamycin for follicular thyroid cancer; 4) Treatment of stage I and II carcinoma of breast, mastectomy vs. lumpectomy; and 5) Phase II evaluation of suramim in advanced stage carcinoma of prostate.

<sup>†</sup>These physicians are full-time Residents in the Laboratory of Pathology.

<sup>\*</sup>This Associate Pathologist spends part time in the activities of the Surgical Pathology Section.

Publications:

- MacLusky JJ, Voit R, Lazo JS, Merino MJ, Schwartz PE. Aromatase activity in human ovarian cancer. *Steroids* 1987;1-12.
- LiVolsi VA, Merino MJ. Pathology of salivary glands. In: Ariyan S, ed. *Cancer of the head and neck*. St. Louis: C V Mosby, 1987.
- Chambers SK, Kapp DS, Lawrence R, Merino MJ, Kohorn EI, Schwartz PE. Prognostic factors and sites of failure in FIGO Stage I, Grade III endometrial carcinoma. *Gynecol Oncol* 1987;27:180-8.
- Merino MJ, Kennedy S, LiVolsi VA, Axiotis C. Spindle cell tumors of the breast. An ultrastructural and immunohistochemical study of 8 cases. *Surg Pathol* 1988;1:193-1.
- Sciocia A, Merino MJ, Haas M, Schwartz PE. Malignant mixed Mullerian tumor of the uterus arising in association with a viable gestation. *Obstet Gynecol* 1988;71:1047-0.
- McIntosh JK, Muli JJ, Merino MD, Rosenberg SA. Synergistic anti-tumor effects of immunotherapy with recombinant interleukin-2 and recombinant tumor necrosis factor. *Cancer Res* 1988;48:4011-7.
- Lefor AT, Merino MJ, Steinberg SM, Roth JA, Pass HI. Computerized tomographic prediction of extraluminal spread and prognostic implications of lesions within in esophageal carcinoma. *Cancer* 1988;62:1287-2.
- Chambers JT, Merino MJ, Kohorn EI, Schwartz PE. Borderline ovarian tumors. *Am J Obstet Gynecol* 1988;159:1088-4.
- Korat O, Trojanowski J, LiVolsi V, Merino M. Antigen expression in normal paraganglia and paragangliomas. *Surg Pathol* 1988;1:33-0.
- Solomon D, Merino MJ. Lymphomatoid granulomatosis mimicking renal cell carcinoma in aspiration cytology. *Surg Pathol* 1988;1:271-5.
- Auerback HE, LiVolsi VA, Merino MJ. Malignant mixed Mullerian tumors of the uterus. An immunohistochemical study. *Int J Gynecol Pathol* 1988;7:123-0.
- Fraker DL, Stovroff MC, Merino MJ, Norton JA. Tolerance to tumor necrosis factor in rats and the relationships to endotoxin tolerance and toxicity. *J Exp Med* 1988;168:95-105.
- Schwartz PE, Merino MJ, McCrea-Curnen M. Rapidly progressively cervical cancer. Clinical management. *Yale J Biol Med* 1988;61:327-38.
- Ohuchi N, Merino MJ, Carter D, Simpson J, Kennedy S, Kufe D, Schlom J. Differential expression of DF3 antigen between papillary carcinomas and benign papillary lesions of the breast. In: Ceriani R, ed. *Immunological approaches to the diagnosis and therapy of breast cancer II*. New York: Plenum Publ Corp (in press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09145-05 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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 NINDS

## COOPERATING UNITS (if any)

Surgical Pathology Section, Pulmonary and Postmortem Section, and  
Ultrastructural Pathology Section, LP, NCI

## LAB/BRANCH

Office of the Clinical Director, NINDS

## SECTION

OCD

## INSTITUTE AND LOCATION

NINDS, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

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

A

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

As described previously, subspecialty expertise in diagnostic neuropathology is provided to the Laboratory of Pathology, NCI, and to all other institutes, via the Office of the Clinical Director, NINCDS. The neuropathology service is integrated with the Surgical Pathology, Postmortem, and the Ultrastructural Pathology Sections. Within the Laboratory of Pathology, both diagnostic (patient care) service and teaching (of pathology residents) are provided. The service also functions in a collaborative manner to provide neuropathological support for a wide range of clinicopathologic investigations.



Major Findings:

The neuropathology service continues to function: (1) to provide a specialized diagnostic service for neurosurgical and autopsy material from NIH patients; (2) to use this material to carry out clinicopathologic studies of primary neurologic disease and neurologic complications of systemic disease, and to (3) teach resident trainees in anatomic pathology the fundamentals of neuropathology; (4) to assist, in collaborative fashion, basic investigators who desire to study human nervous tissue.

Autopsy: As in previous years, the brain was examined in approximately 75% of all autopsies, and approximately one-half of these manifested significant primary or secondary neurologic findings. Neuropathologic consultation is available at the time of autopsy, as needed, for special handling of the brain and/or spinal cord. Detailed and standardized gross examination, description and photography are carried out with the pathology residents at weekly brain cutting sessions. The microscopic slides of all brains and spinal cords are reviewed by the neuropathologist, and the findings integrated into the autopsy report. Presentations of pertinent findings at gross autopsy conference and other clinical conferences are performed by the resident in consultation with the neuropathologist.

Surgicals: Similar to that described previously. Approximately 250 neurosurgical specimens are examined yearly, including both submitted and in-house cases. Approximately 50 intra-operative frozen section consultations are provided yearly. Current in-house case material includes pituitary adenomas, stereotactic biopsies (tumors, AIDS-related lesions), electrocorticographically guided resections for temporal lobe seizures, and PML (progressive multifocal leukoencephalopathy).

Conferences: The case material described above is also utilized for resident teaching conferences and neurology conferences, including presentations at NINCDS Grand Rounds (both formal CPC's and subject reviews).

Specific Studies:

1. Dementia: autopsy confirmation and clinical correlation of patients clinically diagnosed as having Alzheimer's disease (NIA, NIMH, NINCDS).

2. Pituitary adenomas: retrospective study of pituitary adenomas in Cushing's disease.

3. Progressive multifocal leukoencephalopathy (PML): rapid diagnosis/correlation of immunoperoxidase, in-situ hybridization, and EM findings; pathogenesis, including development of an animal model.

4. Malignant gliomas: pathologic study of effects of implanted radiation seeds for malignant gliomas; experimental use of IL2-LAK for brain metastasis.

Publications:

Kinsella TJ, Collins J, Rowland J, Klecker R, Wright D, Katz D, Steinberg S, Glatstein E. Pharmacology and phase I/II study of continuous intravenous infusions of iododeoxyuridine (IdUrd) and hyperfractionated radiotherapy in patients with glioblastoma multiforme. J Clin Oncol (in press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09165-02 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Pulmonary and Postmortem Pathology

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

PI: W.D. Travis Chief, Pulmonary and Postmortem  
Pathology Section

LP NCI.

OTHER: Carl Baker, Peter Howley, David Levens, Lance Liotta, Susan Mackem, Timothy O'Leary, Andrew Larner, Scott Vande Pol, Ann Ginsberg, Gitie Jaffe, Karen Schmidt, Jeffery Taubenberger, David Roth, David Kleiner, Lois Ritchie, Alex Dock, James Rainey, William C. Roberts, and David A. Katz

COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Pulmonary and Postmortem Pathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

20

## PROFESSIONAL:

17

## OTHER:

3

## CHECK APPROPRIATE BOX(ES)

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

A

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

The Section of Pulmonary and Postmortem Pathology, together with the Cytopathology Section, Hematopathology Section, Surgical Pathology Section, and Ultrastructural Pathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all institutes in those investigations which involve the use and study of human pathological material. The Autopsy Suite was recently renovated and new equipment specially designed for safety was developed in conjunction with the Occupational Safety and Health Branch, Division of Safety, Office of the Director. A set of special autopsy safety policies, many of which were developed in our department, is utilized to protect our staff and residents from exposure to tissues contaminated with high risk infectious agents and radiation.

The pulmonary and autopsy pathological material is being actively utilized by the staff and residents for research projects involving clinicopathological correlation and pathological characterization of diseases studied at the Clinical Center. Electron microscopic and immunocytochemical techniques are being applied to study of these diseases. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH. The autopsies from cancer patients treated with interleukin-2 and other forms of immunotherapy have been reviewed and a paper is almost completed on this subject.

Objectives:

The objectives of the Pulmonary and Postmortem Section are: (a) to provide diagnostic services in pulmonary and autopsy pathology for the clinical research projects conducted at NIH. This includes generating pathology reports for current cases and presenting the pathologic findings of autopsies and lung biopsies at clinical conferences with clinicians and radiologists; (b) to carry out independent research; (c) to provide teaching to the NIH residency program in anatomic pathology; and (d) to collaborate with investigators in research involving the use and study of human materials.

The proposed research program includes (a) continuing to provide the services described; (b) increasing interaction with clinical branches in the design and evaluation of protocols; (c) providing opportunities for residents to participate in teaching, and in research projects; (d) developing data retrieval systems for the autopsy and pulmonary pathology material.

Publications:

Travis WD, Li C-Y, Yam LT, Bergstralh EJ, Swee RG. Significance of systemic mast cell disease with associated hematologic disorders. *Cancer* 1988;62:965-2.

Travis WD, Li C-Y, Bergstralh EJ, Yam LT, Swee RG. Systemic mast cell disease: analysis of 58 cases with literature review. *Medicine* 1988;67:345-68.

Skinner M, Pinnette A, Travis WD, Shwachman H, Cohen AS. Isolation and characterization of amyloid protein AA from a patient with cystic fibrosis. *J Lab Clin Med* 1988;112:413-7.

Blute ML, Travis WD, Engen DE, Kvols LK. Primary signet-ring cell carcinoma of the bladder. *J Urol* 1989;141:17-1.

Yeh C-K, Fox PC, Fox CH, Travis WD, Lane HC, Baum BJ. Kaposi's sarcoma of the parotid gland in acquired immunodeficiency syndrome (AIDS) - A case report. *Oral Surg, Oral Path, Oral Med* 1989;67:308-2.

Travis WD, Li C-Y, Bergstralh E. Solid and hematologic malignancies in 60 patients with systemic mast cell disease. *Arch Pathol Lab Med* 1989;113:365-8.

Travis WD, Davis G, Tsokos M, Howley P, Lebowitz R, Merrick HFW, Brady RO, Miller S, Gregg RE, Di Bisceglie AM, Ishak KG, Parker R, Filling-Katz M. Multifocal verruciform xanthoma of the upper aerodigestive tract in a child with systemic lipid storage disease. *Am J Surg Pathol* 1989;13:309-6.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09166-02 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Pathology of Interstitial Pulmonary Fibrosis

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

PI:	W.D. Travis	Chief, Pulmonary and Postmortem Pathology Section	LP NCI
OTHER:	V. Ferrans	Chief, Ultrastructure Section	IR PA NHLBI
	R.G. Crystal	Chief, Pulmonary Branch	IR PB NHLBI

## COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Pulmonary and Postmortem Pathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

A

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

A detailed pathologic review of the NIH (Pulmonary Branch, NHLBI) experience with pulmonary interstitial fibrosis is being performed to investigate potential new approaches to the diagnosis and pathologic subclassification of interstitial fibrosis. Recent reports have described newly recognized forms of interstitial fibrotic lung disease previously classified as idiopathic pulmonary fibrosis suggesting a need for rethinking of traditional concepts of the pathology of pulmonary interstitial fibrosis.

The broad experience of the Pulmonary Branch, NHLBI, provides a rich resource of clinical and pathologic material which may provide the basis for recognition of new prognostically significant forms of interstitial lung fibrosis.

Lung biopsies from 60 patients with idiopathic pulmonary fibrosis have already been reviewed and the data are currently being analyzed. Biopsies from 30 patients with pulmonary histiocytosis X are also being studied. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings.

Publications:

Ognibene FP, Masur H, Rogers P, Travis WD, Suffredini AF, Feuerstein I, Kovacs JA, Gill V, Baird BF, Carrasquillo JA, Parrillo JE, Lane HC, Shelhamer JH. Nonspecific interstitial pneumonitis without evidence of Pneumocystis carinii in asymptomatic patients infected with human immunodeficiency virus (HIV). A bronchoscopic study. *Ann Intern Med* 1988;109:874-9.

Leonard JM, Abramczuk J, Pezen DS, Rutledge R, Belcher JH, Hakim F, Shearer G, Lamperth L, Travis W, Frederickson T, Notkins AL, Martin MA. Development of disease and recovery of transgenic mice containing HIV proviral DNA. *Science* 1988;242:1665-0.

Travis W, Linnoila I, Horowitz M, Pass H, Ozols R, Gazdar A. Pulmonary nodules resembling bronchioloalveolar carcinoma in adolescent cancer patients. *Mod Pathol* 1988;1:372-7.

Travis WD, Lack EE, Ognibene F, Suffredini A, Shelhamer J. Lung biopsy interpretation in the acquired immunodeficiency syndrome (AIDS). Review of the National Institutes of Health experience with literature review. *Prog AIDS Pathol* 1989;1:51-84.

Puri RK, Travis WD, Rosenberg SA. Recombinant interleukin-1 in vivo decreases interleukin-2 or interferon-alpha and interleukin-2 induced increased vascular permeability and edema formation in mice lungs. *Cancer Res* 1989;49:969-6.

Murphy PM, Fox C, Travis WD, Koenig S, Fauci AS. Acquired immunodeficiency may present as severe restrictive lung disease. Report of a case. *Am J Med* 1989; 86:237-0.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00852-36 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Cytology Applied to Human Diagnostic Problems and Research Problems

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

PI:	D. Solomon	Chief, Cytopathology Section	LP NCI
	L. Elwood	Senior Staff Fellow	LP NCI
	T.A. Wood	Biologist	LP NCI
	L. Galito	Biologist	LP NCI
	A.M. Wilder	Biologist	LP NCI
	E. Sanders	Bio. Lab. Tech.	LP NCI
	K. Condron	Microbiologist	LP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Cytopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

7

## PROFESSIONAL:

3

## OTHER:

4

## CHECK APPROPRIATE BOX(ES)

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

A

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

The Cytopathology Section provides complete diagnostic service in exfoliative cytology and fine needle aspiration cytology. The section also routinely applies immunocytochemistry techniques to confirm and/or enhance cytological diagnostic accuracy. In addition, the section collaborates in various clinical research projects utilizing routine microscopy as well as special staining techniques, immunocytochemistry, and flow cytometry.

The fine needle aspiration service is designed to afford maximal flexibility for clinicians and patients. Clinicians may request:

- 1) a pathologist perform the aspiration
- 2) a pathologist instruct the clinician in aspiration technique
- 3) a cytotechnologist assist the clinician in handling the specimen
- 4) aspirations of deep lesions performed by the radiologist with the assistance of a cytotechnologist to evaluate adequacy of the specimen

Major Findings:

Approximately 3800 cytology specimens were evaluated over the past year in the Cytopathology Section, an increase of 10% over previous years. Diagnoses are generally available within 24 hours of receipt. Preliminary diagnoses on STAT cases are communicated within 1-2 hours. Cytology is no longer simply a screening modality: Cytologic evaluation often provides definitive diagnoses which dictate patient care and treatment.

Fine needle aspiration specimens have increased in number by 100% compared to 2 years ago. This modality has been embraced by clinicians as a minimally invasive technique which provides diagnoses rapidly and cost effectively, with minimal discomfort to the patient often obviating more invasive biopsy procedures.

Cases submitted by outside pathologists for consultation by the Cytopathology Section have increased in number by approximately 200% over previous years.

Cytological techniques are utilized in collaborative work with other sections and branches of NIH. For example, single cell tumor suspensions, tumor cell lines, and stimulated lymphocyte cultures are evaluated microscopically and by immunocytochemical techniques.

Publications:

Hamburger JI, Husain M, Nishiyama R, Nunez C, Solomon D. Increasing the accuracy of fine needle biopsy for thyroid nodules. Arch Pathol (in press)

Solomon D, Schwartz A. Renal pathology in von Hippel-Lindau disease. Hum Pathol 1988;19:1072.

Solomon D, Merino M, Linehan M, Jaffe E. Angiocentric malignant lymphoma involving lung and kidney: Mimicking renal cell carcinoma in fine needle aspiration cytology. Surg Pathol 1988;1:273-6.

Yeh C, Fox PC, Ship JA, Busch KA, Bermudez DK, Wilder A, Katz RW, Wolff A, Tylanda CA, Atkinson JC, Baum BJ. Oral defense mechanisms are impaired early in HIV-1 infected patients. J AIDS 1988;1:361-6.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00897-06 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Cytological Diagnosis of Lymphomas by Immunocytochemistry

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

PI:	D. Solomon	Chief, Cytopathology Section	LP NCI
OTHER:	E.S. Jaffe	Chief, Hematopathology Section	LP NCI
	L. Elwood	Senior Staff Fellow	LP NCI
	K. Condron	Microbiologist	LP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Cytopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.5

## PROFESSIONAL:

.25

## OTHER:

.25

## CHECK APPROPRIATE BOX(ES)

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

A

## SUMMARY OF WORK (Use standard un-reduced 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  $\kappa$  and  $\lambda$  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.

Major Findings:

We have investigated 343 specimens, including 188 pleural and peritoneal effusions, 69 cerebrospinal fluids, and 78 fine needle aspiration specimens. We have found 155 cases to be positive for lymphoma and 88 to be reactive in nature. Of the 155 positive cases, 119 were diagnosed as monoclonal B cell proliferations on the basis of either  $\kappa$  or  $\lambda$  light chain but not both. A diagnosis of T cell lymphoma was made in 31 cases on the basis of aberrant marker phenotype or TdT positivity. Acute nonlymphocytic leukemia was diagnosed in two cases and Hodgkin's disease in two cases.

The application of immunocytochemistry to cytology specimens is an extremely valuable adjunct in the diagnosis of hematopoietic malignancies. Definitive cytological diagnosis of relapse/recurrence of disease guides clinical treatment of these patients, often obviating more invasive diagnostic procedures.

References:

Surbone A, Longo DL, DeVita VT Jr, Ihde DC, Duffey P, Jaffe ES, Solomon D, Hubbard SM, Young RC. Residual abdominal masses in aggressive non-Hodgkin's lymphoma after combination chemotherapy: Significance and management. J Clin Oncol 1988;6:1832-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09153-03 LP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Cytophenotypic Analysis of Tumor Suspensions and TIL Cultures in Immunotherapy

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

PI:	D. Solomon	Chief, Cytopathology Section	LP NCI
OTHER:	S. Topalian	Fellow	SB NCI
	S. Rosenberg	Chief	SB NCI
	K. Condron	Microbiologist	LP NCI

COOPERATING UNITS (if any)

Surgery Branch, DCT

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.50

PROFESSIONAL:

.25

OTHER:

.25

CHECK APPROPRIATE BOX(ES)

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

D

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

Clinical trials employing the adoptive transfer of expanded tumor infiltrating lymphocytes to patients with metastatic disease are currently underway under the direction of the Surgery Branch, NCI. Our collaborative effort in this project involves immunocytochemical analysis of tumor cell suspensions to identify (1) the percentage and phenotypic expression of subsets of tumor infiltrating lymphocytes present in the tumor and (2) tumor markers, if any, which are expressed by the tumor cells. Once the tumor infiltrating lymphocyte cultures have been expanded and are to be harvested for patient therapy, we analyze the material using routine cytologic preparations and immunocytochemistry to ensure the cultures are free of tumor cells.

Major Findings:

One hundred ninety-eight tumor suspensions have been evaluated for tumor associated antigens and for phenotypic analysis of tumor infiltrating lymphocytes including: melanomas, renal cell carcinomas and sarcomas.

One hundred sixty-five tumor infiltrating lymphocyte cultures have been examined. In five cases, rare tumor cells were identified. One hundred sixty cultures were free of detectable tumor cells. The majority of reactive lymphoid cells from TIL cultures are Leu-4 positive.

Publications:

Rosenberg SA, Packard BS, Aebersold PM, Solomon D, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. N Engl J Med 1988;319:1676-80.

Topalian SL, Solomon D, Rosenberg SA. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. J Immunol (in press)



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09176-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Quality Assurance in Cervical/Vaginal Cytopathology

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

PI: D. Solomon

Chief, Cytopathology Section

LP NCI

OTHER: J. Smith

Secretary

LP NCI

## COOPERATING UNITS (if any)

DCPC: CDC

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Cytopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.4

## PROFESSIONAL:

.20

## OTHER:

.20

## 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 cervical/vaginal Papanicolaou smear is arguably the most effective cancer screening technique in general use today. However, the accuracy of cervical/vaginal cytopathologic diagnosis has been subject to intense scrutiny and criticism recently.

The Cytopathology Section has been instrumental in organizing three national meetings in 1988 focusing on cervical/vaginal cytology. Two meetings held jointly with the Centers for Disease Control in Atlanta addressed quality assurance measures in cytopathology. The conclusions generated at these meetings are assisting the Health Care Financing Administration in developing and implementing national laboratory quality assurance regulations.

The third meeting consisted of a workshop organized and sponsored by DCPC and DCBD, NCI to develop a uniform system of reporting cervical/vaginal cytopathology. The Bethesda System for Reporting Cervical/Vaginal Cytopathology Diagnoses which resulted from the workshop has received national and international attention. We anticipate that the Bethesda system will supplant the obsolete Papanicolaou classification. Widespread implementation of the Bethesda System will provide for effective communication between laboratories and clinicians well as provide a meaningful data base for research into the biology and epidemiology of cervical neoplasia.

Publications:

Solomon D. Introduction to the proceedings of the conference on the state of the art in quality control measures for diagnostic cytology laboratories. Acta Cytol (in press)

Solomon D, Wied GL. Cervicography: An assessment. J Reprod Med (in press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09177-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Immunocytochemistry of Metastatic Colorectal Carcinoma

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

PI:	L. Elwood	Deputy Chief, Cytopathology Section	LP NCI
OTHER:	M.T. Lotze		SB NCI
	R. Chang	Staff Radiologist	DR CC
	K. Condron	Microbiologist	LP NCI
	A. Wilder	Biologist	LP NCI

## COOPERATING UNITS (if any)

Surgery Branch, DCT, NCI

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Cytopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.25

## PROFESSIONAL:

0.10

## OTHER:

0.15

## 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.)

Studies to assess the effectiveness of combined recombinant IL-2 and anti-tumor antibodies with and without lymphokine activated killer cells (LAK) in treating metastatic cancer are currently underway. We are involved in protocols assessing these therapies in patients with liver metastases from primary colorectal carcinoma. Computed tomography-guided aspiration biopsy specimens are evaluated by a trained cytotechnologist for adequacy in the radiology suite. The procured specimen is then stained in our laboratory by immunocytochemistry with the antibodies L6 and 17-1A. L6 is a murine monoclonal antibody raised against human lung cancer that stains a carbohydrate antigen present on a variety of adenocarcinomas, including most colon cancers. Only scattered cells in normal tissues appear to react with L6. 17-1A is a murine monoclonal antibody generated against a colorectal cell line. Most colorectal cancers react with 17-1A. Some reactivity for 17-1A has been demonstrated in normal colon. Patients are assigned to a protocol according to their immunocytochemical staining pattern.

Major Findings:

Since the first patient was evaluated on 10/26/88, 13 patients have been evaluated for this study. With L6 antibody 2 cases yielded unsatisfactory specimens. Of 11 evaluable cases, 8 (72%) showed positivity. For 17-1A, 2 cases were unsatisfactory. Of the 10 evaluable cases, all were positive. A significant problem is obtaining sufficient viable material from metastatic colon cancer. Immediate evaluation of material for adequacy in the radiology suite has been helpful; however, unsatisfactory specimens are still obtained in some cases. The biopsies are done as outpatient procedures and have been associated with no clinical morbidity. It appears that fine needle aspiration biopsy of metastatic liver lesions yields satisfactory specimens for immunocytochemical characterization in many cases, minimizing morbidity to the patient and cost of tissue procurement.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09178-01 LP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Immunophenotypes of T Cells and Stromal Cells in Mouse Peyer's Patches

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

PI:	L. Elwood	Deputy Chief, Cytopathology Section	LP NCI
OTHER:	G.R. Harriman	Medical Staff Fellow	LCI NIAID
	K. Condron	Microbiologist	LP NCI

COOPERATING UNITS (if any)

Mucosal Immunity Section, NIAID

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.20

PROFESSIONAL:

0.10

OTHER:

0.10

CHECK APPROPRIATE BOX(ES)

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

A

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

The mechanisms involved in B-cell activation in the germinal center are poorly understood. In an attempt to better define some of the factors involved, studies in mice have been underway to characterize the B-cell-associated T-cells in mouse Peyer's patches by flow cytometry and to isolate B-cell associated stromal cells. In our laboratory, we are using frozen section immunohistochemistry to localize the various T-cell subsets found by flow cytometry. For this purpose, dual staining techniques have been utilized. Query stromal cells obtained from tissue culture are being characterized by immunocytochemistry.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 CB 09183-01 LP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) MyoD1 Gene and Protein Expression in Pediatric Tumors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	M. Tsokos	Chief, Ultrastructural Pathology Section
OTHER:	M. Navarro	Guest Researcher
		LP NCI LP NCI
COOPERATING UNITS (if any)  U.S.-Spanish Agreement, Grant No. CCA8309/058		
LAB/BRANCH Laboratory of Pathology		
SECTION Ultrastructural Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects		
<input checked="" type="checkbox"/> (b) Human tissues		
<input type="checkbox"/> (c) Neither		
		B
SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)		
<p>A complementary DNA (cDNA) encoding for the mouse MyoD1 protein was isolated by Dr. A. Lasser and found to induce myogenesis in mesenchymal cells. Polyclonal antisera (rabbit) to bacterial fusion proteins containing the MyoD1 sequence were raised and showed that MyoD1 is a phosphoprotein present in the nuclei of proliferating myoblasts and differentiated myotubes, but not in nonmuscle cell types. The MyoD1 protein was found to contain a domain (22 amino acid) with marked similarity to a region in the myc-family proteins and to the predicted protein products of transcription units in <i>Drosophila</i> which are involved in neuronal determination and the development of nervous system.</p> <p>Based on the above information about the homologies of the MyoD1 protein shared by primitive myoblasts and neural transcription units, and on the knowledge that: (1) certain myogenous tissues (head and neck) originate embryologically from the neural crest, and (2) certain tumors of childhood contain both primitive neural and rhabdomyoblastic components (ectomesenchymoma, medulloblastoma), we will investigate the expression of the MyoD1 gene and protein in the RMS and PNET cell lines, to find out if the MyoD1 gene can be expressed in tissues not expressing a myogenous phenotype, but with the ability to do so. The study will employ Northern and Western blot analysis of the MyoD1 gene and protein correspondingly, and immunofluorescence using the polyclonal antisera against the MyoD1 protein to detect cytologic localization of the protein. The plasmids carrying the MyoD1 gene and antisera will become available to us by Dr. A. Lasser who has already been contacted and agreed to provide us with the material.</p> <p>Moreover, the presence of the MyoD1 protein and the expression of the MyoD1 gene in these RMS cell lines before and after treatment with differentiating agents and TGF-<math>\beta</math> will be evaluated by immunofluorescence, Western blotting and Northern blotting correspondingly, to find out whether the MyoD1 gene and product can serve as a marker of differentiation in RMS.</p>		
229		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09184-01 LP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

P-glycoprotein Expression in Pediatric Cell Tumors

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

PI:	C. Axiotis	Cancer Expert	LP NCI
OTHER:	M. Tsokos	Chief, Ultrastructural Pathology Section	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3/4

PROFESSIONAL:

3/4

OTHER:

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.)

P-glycoprotein has been associated with a multidrug-resistant (MDR) phenotype in a wide variety of animal and human tumor cell lines and clinical tumor specimens. Pediatric tumors, however, have not been included in the studies reported so far. Given the known chemosensitivity of certain solid pediatric tumors, such as Ewing's sarcoma, primitive neuroectodermal tumor and rhabdomyosarcoma, to the point that all clinical trials use chemotherapy and radiotherapy as primary methods of treatment, it is important to detect levels of P-glycoprotein in these tumors before and after treatment.

Tissues from Ewing's sarcomas, primitive neuroectodermal tumors and rhabdomyosarcomas are available from patients included in the clinical therapeutic protocols in the Pediatric Oncology Branch of NIH. All these tumors are well characterized and classified histologically by light and electron microscopy. Moreover, tissues from primary sites before treatment and from metastatic sites or sites of recurrence after treatment are available in many cases. All these tissues will be evaluated for the presence of P-glycoprotein using a commercially available antibody against P-glycoprotein and employing a modified immunoperoxidase "sandwich" staining method (Chan et al., Lab. Invest. 59: 870, 1988). The results of immunostaining for P-glycoprotein will be correlated with clinical data of patient outcome and response to treatment.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09188-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Flow Cytometric Analysis of Childhood Sarcomas

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

PI:	C. McLeod	Biotechnology Fellow	LP NCI
OTHER:	M. Tsokos	Chief, Ultrastructural Pathology Section	LP NCI
	M. Horowitz	Senior Investigator	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:

## CHECK APPROPRIATE BOX(ES)

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

A

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Hyperdiploidy has been associated with an unfavorable histology (anaplastic) in Wilms' tumor and with a better response to chemotherapy in unresectable neuroblastoma. Moreover, DNA-aneuploidy was found in rhabdomyosarcoma (RMS), in contrast to Ewing's sarcoma and primitive neuroectodermal tumor (PNET) in a recent report using a limited number of tumors. The latter was suggested to be an additional criterion in the differential diagnosis of childhood sarcomas.

We will analyze the cellular DNA content of 100 tumor samples including RMS, PNET, and Ewing's sarcoma using histologic sections (10 or more per tumor sample) from paraffin blocks. DNA histograms will be used to determine: (1) the number of malignant stem lines in each sample; (2) the DNA index of the stem line(s) (i.e., ratio of the modal G<sub>0</sub>/G<sub>1</sub> channel for tumor cells versus that of normal diploid cells) and (3) the percentages of tumor cells in S phase and G<sub>2</sub>+M phase. The findings will be compared with the histology of the tumors and the histologic subtypes in the case of RMS (embryonal vs. alveolar, and the newly defined by us histologic subtype with aggressive clinical outcome, the solid variant alveolar RMS). Given the fact that all patients were treated homogeneously according to clinical protocols, meaningful comparisons between DNA-ploidy and clinical outcome or response to treatment will be made as well.

The findings will help us draw conclusions as to a possible prognostic or diagnostic role of flow cytometry in this group of pediatric sarcomas.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09172-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Tumor Cell Interactions with Thrombospondin

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

PI:	D.D. Roberts	Acting Chief, Biochemical Pathology Section	LP NCI
OTHER:	V. Zabrenetzky	Biotechnology Fellow	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:

1.3

## PROFESSIONAL:

1.3

## OTHER:

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 functions of thrombospondin in tumor cell adhesion and migration are being investigated. We have identified two regions of the thrombospondin molecule that mediate adhesive and migratory responses of cultured human melanoma cells to thrombospondin. The carboxyl-terminal domain mediates attachment and haptotaxis, while the amino-terminal domain mediates cell spreading and chemotaxis. The cell receptors recognizing these two regions of thrombospondin are under investigation. One class of receptors are sulfated glycoconjugates which bind to the amino-terminal domain of thrombospondin. A minor heparan sulfate proteoglycan that binds thrombospondin with high affinity was identified in two melanoma cell lines and purified by affinity chromatography on thrombospondin-Sepharose (Cancer Res. 48: 6875, 1988). Monoclonal antibodies to this proteoglycan will be prepared and used to examine the function of this binding. An unusual sulfated glycolipid present only in melanoma cell lines that spread on thrombospondin was also found to bind thrombospondin (ibid.). This glycolipid purified from peripheral nerve and a monoclonal antibody to the glycolipid specifically inhibit melanoma cell spreading on thrombospondin but not on fibronectin. To further define the mechanism of thrombospondin interactions with tumor cells, receptors for the carboxyl-terminus of thrombospondin will be characterized. The intracellular responses of cells to binding of thrombospondin to the two types of receptors are also being investigated.

Major Findings:

1) Sulfated glycoconjugates produced by human melanoma cell lines were identified by metabolic labeling with [<sup>35</sup>S]-sulfate and tested for binding to thrombospondin. Heparan sulfate proteoglycans that bind thrombospondin were made by both spreading and nonspreading cell lines. Thrombospondin bound with high affinity to a minor heparan sulfate proteoglycan but not to the major chondroitin sulfate. The active heparan sulfate proteoglycan could be purified by affinity chromatography on thrombospondin-agarose or hydrophobic interaction on octyl-Sepharose. Binding required the amino terminus of TSP based on antibody and proteolysis studies. [<sup>35</sup>S]-labeled glycolipids were also isolated from the melanoma cell lines. Sulfated glucuronosylparagloboside was found in three cell lines that spread on thrombospondin but was absent in a nonspreading line. Thrombospondin bound to the isolated glycolipid and the glycolipid and an antibody to this structure inhibited spreading on thrombospondin but not on fibronectin substrates. Thus, the presence of glycoconjugates with terminal nonreducing glucuronosyl 3-sulfate correlates with melanoma cell spreading on thrombospondin, whereas expression of heparan sulfate proteoglycans that bind thrombospondin does not.

2) Cellular responses to binding of thrombospondin to the two classes of receptors are being investigated. Changes in intracellular inositol phosphate and cyclic AMP were observed following exposure of melanoma cell monolayers to thrombospondin. Experiments are in progress to determine levels of these during attachment of melanoma cells to thrombospondin substrates.

Publications:

Roberts DD. Interactions of thrombospondin with sulfated glycolipids and proteoglycans of human melanoma cells. *Cancer Res* 1988;48:6875-93.

Furukawa K, Roberts DD, Endo, T, Kobata A. Structural study of the sugar chains of human platelet thrombospondin. *Arch Biochem Biophys* 1989;270:302-12.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09173-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Carbohydrate Receptors for Human Pathogens

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

PI: D.D. Roberts Acting Chief, Biochemical Pathology Section LP NCI

## COOPERATING UNITS (if any)

L. Olson and M. Barile, Center for Drugs and Biologics, FDA  
 T. Hackstadt, Dept. of Pathology, University of Texas Medical Branch, Galveston  
 T. Walsh, Pediatric Oncology, NCI, NIH

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Biochemical Pathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

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) Mycoplasma pneumoniae: We have found that sulfated glycolipids and  $\alpha$ 2-3-linked sialyl glycoproteins are two independent receptors for cytoadhesion of M. pneumoniae and that the former is present at high concentrations in human trachea which is the primary site of infection. Monoclonal antibodies to the pathogen will be used to define receptors for these, and the respective carbohydrates on affinity matrices will be used to purify their receptors. To date, the extended specificity of the sialyloligosaccharide binding is only partially defined. Additional oligosaccharides will be prepared and their structures defined to complete this analysis. The ability of these carbohydrates to promote chemotaxis of M. pneumoniae will also be tested.

2) Chlamydia trachomatis: Several serotypes of Chlamydia were examined for binding to glycolipids and glycoproteins. Three binding specificities were identified from these studies. The elementary bodies bind specifically to glycolipids containing GalNAc $\beta$ 1-4Gal $\beta$ 1-4Glc sequences, heparin or fucoidin conjugated to albumin, and to the adhesive proteins fibronectin and laminin. Inhibitors of each of these specificities will be used to determine the role of each in infection of cultured cell monolayers.

3) Fungal pathogens: Carbohydrate binding specificity of C. albicans and several other human dermatophytes will be examined.

Major Findings:

Several purified glycoproteins including laminin, fetuin, and human chorionic gonadotropin promote a dose-dependent and saturable adhesion of Mycoplasma pneumoniae when adsorbed on plastic. Adhesion to the proteins is energy dependent as no attachment occurs in media without glucose. Adhesion to all of the proteins requires sialic acid, and only those proteins with  $\alpha$ 2-3-linked sialic acid are active. The  $\alpha$ -subunit of human chorionic gonadotropin also promotes attachment, suggesting that a simple biantennary asparagine-linked oligosaccharide is sufficient for binding. Soluble laminin and 3'-sialyllactose but not 6' sialyllactose inhibit attachment of M. pneumoniae to laminin. M. pneumoniae also adhere to sulfatides adsorbed on plastic. Dextran sulfate, which inhibits M. pneumoniae attachment on sulfatide, does not inhibit attachment on laminin and 3'-sialyllactose does not inhibit attachment on sulfatide, suggesting that two distinct receptor specificities mediate binding to these two carbohydrate receptors. Both 3'-sialyllactose and dextran sulfate partially inhibit M. pneumoniae adhesion to a human colon adenocarcinoma cell line (WiDr) at concentrations which completely inhibit binding to laminin or sulfatide, and in combination they inhibit binding of M. pneumoniae to these cells by 90%. Thus, both receptor specificities contribute to M. pneumoniae adhesion to cultured human cells.

Publications:

Roberts DD, Olson LD, Barile MF, Ginsburg V, Krivan HC. Sialic acid-dependent adhesion of Mycoplasma pneumoniae to purified glycoproteins. J Biol Chem (in press)

Krivan HC, Olson LD, Barile MF, Ginsburg V, Roberts DD. Adhesion of Mycoplasma pneumoniae to sulfated glycolipids and inhibition by dextran sulfate. J Biol Chem (in press)



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09174-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Role of Sulfated Glycoconjugates in Tumor Cell Adhesion

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

PI: D.D. Roberts Acting Chief, Biochemical Pathology Section LP NCI  
 OTHER: V. Zabrenetzky Biotechnology Fellow LP NCI

## COOPERATING UNITS (if any)

JoAnne Cashel, Biochemical Pathology Section, LP, NCI

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Biochemical Pathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.3

## PROFESSIONAL:

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1.0

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 (a1) Minors  
 (a2) Interviews

B

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

We have found that sulfated glycolipids participate in tumor cell adhesion by directly promoting adhesion (Cancer Res. 48: 3367, 1988) and as receptors for thrombospondin on melanoma cells (Cancer Res. 48: 6785, 1988). Relatively few of the glycolipids belonging to this class have been characterized. We are purifying novel sulfated glycolipids from several sources including human kidney and meconium and breast and small cell lung carcinoma cell lines. The structures of these glycolipids will be examined using chemical and immunological approaches. Monoclonal antibodies to these will be used to examine the potential of these structures as tumor markers. To further characterize the effects of sulfated glycolipids on tumor cell behavior, we are also examining the intracellular responses following attachment of melanoma cells on sulfatide-coated substrates.

Major Findings:

A novel sulfated glycosphingolipid belonging to the globo series was isolated from human kidney. The lipid was isolated from pooled kidney by chloroform/methanol extraction, alkali treatment, DEAE-Sephadex and silicic acid column chromatography and preparative thin layer chromatography. Based on infrared and two-dimensional proton magnetic resonance spectroscopy, negative secondary ion mass spectroscopy, methylation analysis, and interaction with monoclonal antibodies and sulfatide-binding proteins, the structure is proposed to be HSO<sub>3</sub>-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 ceramide. This glycolipid reacts with a monoclonal antibody SSEA-3 on thin layer chromatograms and by radioimmunoassay and binds <sup>125</sup>I-labeled thrombospondin. The yield of glycolipid was 0.19 nmol/g of kidney.

Publications:

Nagai K, Roberts DD, Toida T, Kushi Y, Handa S, Ishizuka I. Sulfated globo-pentaosylceramide from human kidney. J Biol Chem (in press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

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Glycolipid Antigens Expressed in Esophageal Carcinoma

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

PI:	D.D. Roberts	Acting Chief, Biochemical Pathology Section	LP NCI
OTHER:	W. Wang	Guest Worker	LP NCI

## COOPERATING UNITS (if any)

L. Zhang and V. Ginsburg, LSB, NIDDK, NIH

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Biochemical Pathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.2

## OTHER:

0.8

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 (a1) Minors  
 (a2) Interviews

B

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

Esophageal carcinoma is endemic in the Ho Nan Province in China. Characterization of antigens unique to these tumors would be useful for diagnosis of this carcinoma. Glycolipids isolated from esophageal tissues obtained from 11 cancer patients, 10 normal individual controls, and a cultured esophageal carcinoma line were examined using several monoclonal antibodies. Several glycolipids differ in amount between the tumor samples and normal controls. Both neutral and sialylated glycolipids containing 3-fucosyllactosamine sequences differ in expression between the tumor and control tissues and a heptaglycosylceramide reacting with antibodies to this sequence is found only in the tumor tissues. The same glycolipids are detected in the cultured cell line, confirming that the tumor antigen is produced by the tumor and is not due to contamination by infiltrating granulocytes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

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Stimulated Motility in Tumor Cells

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

PI:	E. Schiffmann	Research Chemist	LP NCI
	L. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
OTHER:	M.L. Stracke	Biotechnology Fellow	LP NCI
	E.C. Kohn	Medical Staff Fellow	MB NCI
	M. Beckner	Biotechnology Fellow	LP NCI
	S. Aznavoorian	Guest Worker	LP NCI
	R. Guirguis	Guest Researcher	LP NCI
	H. Krutzsch	Expert	LP NCI

COOPERATING UNITS (if any) M. Seiki Visiting Scientist LP NCI

C.E. Myers, Medicine Branch, NCI; M.M. Rechler, MCNE, NIDDK

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

7.3

6.3

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 purification of melanoma autocrine motility factor (AMF), a protein of considerable significance in metastasis, has proceeded to a stage where AMF shows a single band on silver stain-visualized gel electrophoresis. A series of chromatographic steps resulted in the isolation of homogeneous material which activity was completely inhibitable by the action of pertussis toxin (PT). The material is being processed for N-terminal sequence determination. In mechanistic studies, a membrane-containing fraction from melanoma cells contained a substrate for the ADP-ribosylation reaction induced by PT. In the intact cell nicotinamide partially reversed the inhibitory action of PT upon AMF-induced cell motility. In membrane fractions AMF stimulated the formation of inositol trisphosphate from exogenous phosphatidyl inositol diphosphate via a phospholipase C reaction, supporting a role for this enzyme in signal transduction of AMF-stimulated motility. In addition to AMF, the insulin peptides (IGFs) induced motility in melanoma cells. A receptor for IGF-I was demonstrated in these cells with the aid of a specific antibody. Other tumor cells, including carcinomas responded to the IGFs which also induced mitogenesis in some cell lines. In IGF-I-stimulated cells, glycolysis was shown to be the principal source of energy for motility. The melanoma cells could adhere and migrate to extracellular matrix components such as type IV collagen and laminin. Chemotaxis but not adherence to these agents was inhibited by PT, suggesting different receptors and transduction pathways for each response. In a clinical study the AMF contents of bladder cancer patient urines were correlated with the severity of disease, results promising for non-invasive adjunctive diagnosis of this cancer.



Major Findings:A) Melanoma Cells

## I) Autocrine Motility Factor

1) Purification of autocrine motility factor (AMF)

We have progressed in the purification of AMF from concentrated conditioned media of A2058 human melanoma cells. A 200-liter volume of the media was concentrated 500-fold with an Amicon filter that retained molecules with an Mr  $> 30$  KD. After washing to remove dialysable materials, the concentrate was chromatographed on a phenylsepharose hydrophobic interaction column using a reversed ammonium sulfate gradient in the presence of 30% ethylene glycol. After bioassay of the fractions from this procedure, the active material was applied to an anion exchange column using a salt gradient in ethylene glycol. A final anion exchange procedure with active material from the previous separation resulted in a preparation that after electrophoresis under non-reducing conditions gave a single band upon silver staining. The estimated Mr range was 56-60 KD. When run under reducing conditions, the material migrated as a doublet or triplet with an Mr range of 62-68 KD. Variable glycosylation of the protein might account for these observations. The AMF activity at this stage of purification was completely inhibited by pertussis toxin. This property is characteristic of the cytokine throughout the isolation procedure.

2) Signal transduction: mechanism of action of AMF

We have previously shown that AMF stimulated an increase in the formation of inositol-tris-phosphate (IP<sub>3</sub>) in human melanoma cells. This compound is believed to be a critical agent for mobilizing intracellular Ca<sup>++</sup>, which in turn is likely to play a role in the activation of the cytoskeleton and a subsequent motile response. Recently, we have prepared membrane-containing fractions of melanoma cells. This preparation was shown to contain a substrate for ADP-ribosylation in the presence of pertussis toxin (PT). Previously, PT was shown to inhibit markedly AMF-stimulated motility in melanoma cells. We also observed that in whole cells pretreated with PT, the continuous presence of either nicotineamide or N-methyl nicotineamide (50 mM), the PT effect was partially inhibited. This effect was probably the result of 'product inhibition' of the ADP-ribosylation reaction and underscores the action of PT in inhibiting the mediation of motility by a G-protein. We also showed that in the membrane preparation, AMF stimulated a phospholipase C-specific hydrolysis of (<sup>3</sup>H) phosphatidyl inositol diphosphate to yield (<sup>3</sup>H) IP<sub>3</sub>.

## II) Other Agents Stimulating Motility

a) Insulin-like growth factors(1) The IGF-I receptor as a mediator of motility

We have previously demonstrated that insulin peptides stimulated chemotaxis in human melanoma cells. The order of efficacies was: insulin-like growth factor-I (IGF-I)  $>$  insulin-like growth factor 2 (IGF-II)  $>$  insulin. Evidence

was presented for a high affinity IGF-I receptor ( $K_d \sim 10^{-10}$ ) with about 5000 receptors per cell. The peptide-stimulated chemotaxis was not inhibited by pertussis toxin. We have now found that the antibody to the IGF-I receptor,  $\alpha$ IR-3, inhibited both the binding of  $^{125}\text{I}$ -IGF-I to the melanoma cells and the motile response of those cells to IGF-I. However, the antibody did not inhibit AMF-stimulated motility. Cross-linking of  $^{125}\text{I}$ -IGF-I to the cells yielded a protein that migrated with an  $M_r \sim 130$  KD on SDS-PAGE, corresponding to authentic IGF-I receptor. Mixtures of IGF-I and AMF produced a motility response that was greater than that stimulated by either agent alone. The results suggested that IGFs and AMF act through different receptors and signal transducer mechanisms. The metastatic capacity of the cell would appear to be enhanced by its ability to respond to multiple motility-inducing stimuli acting through different effector pathways.

## (2) Stimulation of energy mobilization by IGF-I

We have found that IGF-I stimulated metabolism of glucose in melanoma cells at peptide concentrations that elicited a strong motility response. This response depends upon the level of glucose in the media. At optimal levels of glucose, the stimulated metabolism of labelled glucose was through glycolysis. At low levels or in glucose-free media, the motile response was diminished by 65% and glucose metabolism was shifted toward the tricarboxylic acid cycle. Under these conditions, motility was markedly reduced by oligomycin, an inhibitor of mitochondrial respiration. On the other hand, at levels of glucose optimal for motility, oxamate, an inhibitor of glycolysis, markedly depressed stimulated motility, while oligomycin had no effect on cell migration. The results indicate that a highly malignant tumor cell depends upon glycolytic sources of energy for motility. However, in a glucose-deficient environment, the cell can shift to the tricarboxylic acid cycle for energy in generating a motile response.

## II b) Extracellular matrix components (ECM)

ECM components have previously been shown to play roles in the adherence of tumor cells to biological barriers such as basement membranes. The effects of both the soluble and insolubilized matrix components upon cell motility and attachment was studied respectively for type IV collagen (IV), laminin (LAM) and fibronectin (FN). Attachment was observed as 'haptotaxis', the movement of cells onto a bound attractant. The chemotactic responses (soluble attractant) of melanoma cells to IV and LAM were substantial and were inhibited by pretreatment of the cells with pertussis toxin (PT). On the other hand, the haptotactic responses to these ligands were not inhibited by PT. Neither the chemotactic nor the haptotactic response to FN, however, was affected by PT. The peptide GRGDS, known to simulate a binding site on FN, inhibited both the chemotactic and haptotactic responses to FN but had no effect upon these responses to IV or LAM. These findings indicate that the receptor for FN is distinct from those for IV and LAM and that for the latter two ECM components, chemotaxis and haptotaxis may be induced through separate transduction mechanisms. Also, for a given ECM component, the differential response to a haptotactically or chemotactically presented stimulus could be exercised through separate domains of the molecule acting upon the same or different receptors. Such

putative domains might exist separately in enzymatically produced fragments of the ECM and play a role in the cell's traversing basement membranes.

## II c) Antibodies to melanoma receptors

A project has been initiated to obtain antibodies to receptors to ligands which stimulate motility and adherence in melanoma cells. The procedure involves the injection of intact human melanoma cells (A2058) into mice and, after an interval, isolating splenic lymphocytes from these immunogenically challenged animals. The cells were then fused with myeloma cells in a typical hybridoma generation procedure. Cloning procedures yielded colonies whose media were chromatographically processed to obtain IgG monoclonal components. A number of these were tested for their effects upon A2058 motility responses to AMF, LAM, and IV collagen. Of 13 clones tested, one appeared to be quite promising, having a strong inhibitory effect against IV-stimulated motility. LAM-stimulated motility was inhibited to a lesser extent, as was the AMF-induced response. In general, the effective dilution range for a given antibody effect was narrow. Plans to obtain antibodies of higher titre include a recloning of the promising preparations. Growing the hybridomas in the mouse peritoneal cavity should enhance the yield of monoclonal antibodies (mAbs). Ouchterlony analysis will be used to determine the subclass of the mAbs. Eventually, the use of high titre mAbs would be used for isolation of membrane receptors. Cells labelled with  $^{125}\text{I}$  or  $^{14}\text{C}$ -leucine could be fractionated to obtain membrane fragments from which putative receptors would be extracted and then subjected to immunoprecipitation procedures with the mAbs.

## B) Other Cells Responding to Various Ligands

### I) Stimulation of Motility and Mitogenesis in Tumor Cells by Insulin Peptides

The migration and mitogenic responses to the insulin peptides were examined in a number of neoplastic cells. Insulin (Ins) (100 nM) was found to induce motility in the carcinoma cell lines: MDA-231 (breast), T24 A & B (bladder), and OVCAR-3 (ovarian). The extents of the responses were about 50% of that induced by AMF (melanoma). However, a response equivalent to that induced by AMF was caused by Ins in the 5R line (RAS, rat embryo fibroblast). Ins in these cells produced both chemokinetic (CK) and chemotactic (CT) responses. Insulin-like growth factor I (IGF-I) also stimulated responses in cells that were less than 40% of the AMF-induced responses. IGF-II induced a maximal response in the carcinoma lines that was equivalent to the AMF-induced responses. AMF stimulated pertussis-toxin (PT) inhibitable responses in all lines except in OVCAR. PT did not inhibit insulin peptide-stimulated motility in any cell line tested. IGF-I and II were mitogenic for the 5R and A2058 (human melanoma) lines but not for the carcinoma lines. The insulin peptides, secreted by a variety of normal tissues and certain tumors, may be important homing and mitogenic agents for tumor cells during the metastatic cascade.

## II) Identification of an AMF from 3T3 RAS Cells

Metastatic 3T3 RAS cells have been found to produce an AMF that had an estimated Mr ~ 100 KD from gel filtration data. The material appears to be a protein that is inactivated by heating above 60°C. The active material was partially purified by the following sequence of steps: amicon filtration with a filter that retained molecules > 30 KD; DEAE anion exchange chromatography; and gel electrophoresis under non-denaturing conditions. The new AMF induced motility in both 3T3 and A2058 cells, as well as in the producer cells. In contradistinction to the A2058 AMF, these responses were not inhibited by PT. Based upon its physical properties, stability to heating, and the lack of PT sensitivity of its stimulated response in cells, the 3T3 RAS-derived AMF is distinct from the melanoma cell AMF. These findings emphasize the versatility of metastatic cells in having at their disposal a variety of receptors and transducer pathways to generate a motile response. These characteristics could well enhance metastatic potential of tumor cells.

## C) Applications of Autocrine Motility Studies

We have utilized the motility assay to detect the presence of AMF in the urine of bladder cancer patients. In a comparison of the processed 24 hr urine samples of 22 transitional bladder cell carcinoma patients (TCC) with those from 27 patients with non-malignant diagnoses, it was found that significantly higher values of AMF were present in the TCC patient urine samples than in the urines from non-malignant cases. In follow-up screening studies evaluating TCC recurrence, the urine from patients with recurring tumors had significantly higher motility stimulating activity for the responder cells than did the urines from tumor-free patients. In addition, an ELISA procedure confirmed the motility assays. We also found that a cultured line of bladder cancer cells, T24P, produced a strong motility response to its own conditioned media. This result is consistent with the source of urinary AMF as a bladder tumor itself. We suggest that measurement of urinary AMF may be a reliable diagnostic marker for bladder cancer.

## Publications:

Guirguis R, Schiffmann E, Liu B, Birkbeck D, Engel J, Liotta L. Detection of autocrine motility factor(s) in urine as markers of bladder cancer. *J Natl Cancer Inst* 1988;80:1203-1.

Stracke ML, Kohn EC, Aznavoorian SA, Wilson LL, Salomon D, Krutzsch HC, Liotta LA, Schiffmann E. Insulin-like growth factors stimulate chemotaxis in human melanoma cells. *Biochem Biophys Res Commun* 1988;153:1076-3.

Liotta LA, Schiffmann E. Tumour motility factors. In: Hart I, ed. *Cancer surveys - advances & prospects in clinical, epidemiological and laboratory oncology*, vol. 7, No. 4. London: Oxford Univ. Press, 1988;631-52.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00892-06 LP

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## 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	Special Staff Fellow	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
	H.C. Krutzsch	Expert	LP NCI
	V. Cioce	Guest Researcher	LP NCI
	A. Rosengard	Biotechnology Fellow	LP NCI
	A. Leone	Guest Researcher	LP NCI

## COOPERATING UNITS (if any)

Molecular Oncology Incorporated, Gaithersburg, MD (CRADA)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Tumor Invasion and Metastases Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.8

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2.8

## 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 molecular biology of tumor metastasis has been investigated. The NM23 gene was identified on the basis of its consistent down regulation in high metastatic potential tumor cells in four rodent experimental systems, as compared to related low- or nonmetastatic counterparts. NM23 RNA levels were also decreased in human infiltrating ductal breast carcinomas with histopathological evidence of high metastatic potential. The NM23 gene represents the first non-immunologically related gene associated with low tumor metastatic potential. It is of potential diagnostic and therapeutic importance.

A 17 kDa NM23 gene product has been identified, based on the DNA sequence of a murine pNM23-1 cDNA clone, hybrid selection experiments and Western blots using affinity purified rabbit antisera to two predicted NM23 peptides. NM23 protein levels were down-regulated in high metastatic potential K-1735 TK cells compared to related, low metastatic potential K-1735 clone 19 cells. NM23 protein is found in both nuclear and cytoplasmic subcellular fractions. The predicted NM23 protein sequence is 95% homologous to a Drosophila gene, awd, which is critical to normal development.

A human NM23 cDNA clone has been identified, sequenced, and subcloned into bacterial expression vectors. Recombinant protein is being used to prepare monoclonal antibodies.

Preliminary transfection experiments suggest that addition of NM23 and a SV40 early promoter to high metastatic potential murine K-1735 melanoma cells may reduce primary tumor size and the development of pulmonary metastases. Further confirmatory experiments are necessary before a final conclusion can be drawn on the anti-metastatic activity of the NM23 gene.

### Major Findings:

The NM23 gene was identified by differential colony hybridization using RNA from related low and high metastatic potential murine melanoma cell lines as probes. NM23 RNA levels have been determined in four rodent metastasis model systems: (1) murine K-1735 melanoma cell lines, in which the gene was identified; (2) rat nitrosomethylurea-induced mammary tumors; (3) mouse mammary tumors induced by two strains of mouse mammary tumor virus; (4) rat embryo fibroblasts transfected with c-Ha-ras or cotransfected with ras + adenovirus 2 Ela. In each case, non- or low metastatic tumor cells contained higher NM23 RNA levels than did related, high metastatic tumor cells. In addition, low NM23 RNA levels were observed in human primary infiltrating ductal carcinomas from patients with lymph node metastases, as well as a proportion (25%) of carcinomas from patients without evidence of metastasis at surgery. The NM23 gene is therefore differentially expressed in a human cancer, and may be of diagnostic importance. The NM23 gene represents the first non-immunologically related tumor cell gene identified which is associated with low metastatic potential. It is a candidate for a gene that suppresses the malignant phenotype.

### Identification and Characterization of a NM23 Gene Product

The DNA sequence of a murine pNM23-1 cDNA clone was previously identified. The sequence contains a single open reading frame extending to its 5' terminus, and four methionines, one of which is surrounded by an optimal translation initiation sequence. The DNA sequence from this putative initiating methionine to contiguous double stop codons would encode a 17 kDa protein. Hybrid selection experiments using the pNM23-1 cDNA insert and murine K-1735 clone 19 RNA indicate two gene products of 17 and 19 kDa, the former in agreement with the DNA sequence data. Peptides corresponding to three regions of the pNM23-1 predicted sequence were synthesized, and affinity purified rabbit antisera prepared to them: peptide 10 corresponds to the predicted N terminus of the 17 kDa NM23 protein; peptide 11 includes a hydrophilic segment of the predicted protein; and peptide 65 corresponds to the predicted 5' untranslated region. Antibodies to peptides 10 and 11 recognized a 17 kDa in lysates of murine K-1735 melanoma clone 19 cells on Western blots, in agreement with DNA sequence and hybrid selection data. The 17 kDa band was specific, in that a molar excess of peptide inhibited the antibody-antigen reaction, and an affinity purified antibody to a peptide unrelated to NM23 did not identify this band. Anti-peptide 65 antibody, while recognizing a BSA-peptide 65 complex, did not identify a protein band from clone 19 lysates, confirming the predicted initiating methionine in the pNM23-1 sequence. Thus, affinity purified anti-peptide antibodies have been generated to one of two NM23 gene products.

We have determined that NM23 protein levels are also differentially expressed with tumor metastatic potential in the murine K-1735 melanoma system. Low metastatic potential clone 19 cells contained significantly greater 17 kDa NM23 proteins on Western blots than the related, high metastatic potential K-1735 TK cell line. Current efforts are aimed at investigating NM23 protein levels in human tumors and tumor cell lines.

The predicted pNM23-1 DNA sequence contained three leucines in the arrangement of a leucine zipper, with a 5' basic amino acid region, suggesting that the NM23

protein may be regulatory. While leucine zippers of this size have not yet been confirmed, mutation analysis indicates that three leucine zippers in other regulatory proteins were functional. In support of this possibility, NM23 protein has been localized to both the nuclear and cytoplasmic subcellular fractions.

Using a Swiss protein database search, the 17 kDa NM23 protein has been shown to be 95% homologous to a recently identified Drosophila developmental gene, abnormal wing disk (awd). Mutant forms of awd are characterized by a reduction in awd RNA and a 17 kDa protein, in agreement with NM23 data in metastasis, and gross developmental changes similar in nature to metastasis: Altered cell morphology in the imaginal wing disks and lack of differentiation in the wings, eyes, ovaries, antennae and brain. The NM23 protein is highly conserved in evolution, and may have a critical role in normal development.

#### Human NM23 Gene

In a previous year a human NM23 cDNA clone was identified in collaboration with Molecular Oncology, Incorporated. The DNA sequence has been determined, and the predicted amino acid sequence is 94% identical to the murine 17 kDa NM23 protein. The cDNA insert has been subcloned into a bacterial expression vector, and the recombinant protein is being used to develop monoclonal antibodies to NM23 proteins.

A second, distinct human cDNA clone cross hybridizing to the pNM23-1 clone has also been isolated, and is currently being characterized.

#### Function of the NM23 Gene

In preliminary experiments high metastatic potential, murine K-1735 melanoma TK cells were transfected with pNM23-1 and pRSVneo or as a control, pRSVneo. Five randomly selected neomycin resistant lines from the pNM23-1 and pRSVneo (experimental) and pRSVneo (control) plates were selected and characterized. An additional weak band of NM23 RNA was present in the experimental lines. When injected into mice intravenously in two experiments, the experimental lines produced 67 and 22% fewer pulmonary metastases than the control lines. Upon subcutaneous injection (n = 1), the experimental lines formed smaller primary tumors and 70% fewer metastases than the control lines. These data suggest that the NM23 gene may have suppressor activity for primary tumor formation and/or metastasis. Considerable additional testing is required to confirm these preliminary results. Current efforts are directed towards the development of a better pNM23 construct, in the hope of obtaining higher expression of the transfected gene. Also, an improved control construct, containing the SV40 early promoter but not the NM23 insert, has been developed and will be tested.

Publications: .

Kalebic T, Williams JE, Talmadge JE, Kao-Shan, C-S, Kravitz B, Locklear K, Siegal GP, Liotta LA, Sobel ME, Steeg PS. A novel method for selection of invasive tumor cells. Derivation and characterization of highly metastatic K-1735 melanoma cell lines based on in vitro and in vivo invasive capacity. Clin Exp Metast 1988;6:301-18.

Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA, Sobel ME. Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 1988;80:200-3.

Steeg PS, Bevilacqua G, Pozzatti R, Liotta LA, Sobel ME. Expression of NM23, a gene associated with low tumor metastatic potential, is increased during adenovirus 2 Ela inhibition of experimental metastasis. Cancer Res 1988;48: 6550-4.

Steeg PS, Bevilacqua G, Rosengard A, Sobel ME, Cioce V, Liotta LA. Altered gene expression in tumor metastasis: The NM23 gene. In: Schirmacher V, Schwartz-Albiez R, eds. Cancer metastasis. Molecular and cellular biology, host immune responses and perspectives for treatment. Heidelberg: Springer-Verlag. (in press)

Steeg PS. The search for metastasis suppressor genes. Invasion and Metastasis (special volume). (in press)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00893-06 LP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

DNA Mediated Transfer of Metastatic Potential

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

PI:	L.A. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI
OTHER:	W.G. Stetler-Stevenson	Senior Staff Fellow	LP NCI
	D.L. Levens	Chief, Gene Regulation Section	LP NCI
	P.S. Steeg	Special Staff Fellow	LP NCI

COOPERATING UNITS (if any)

S. Garbisa, Padova Institute of Histology, Italy

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B

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

Activated ras oncogene transfection into suitable recipient cells has been shown to induce the metastatic phenotype. However, when the cooperating oncogene Ela was used in conjunction with ras<sup>H</sup>, metastasis was only rarely seen. We have used this model system to study the correlation of basement membrane collagenolysis with metastatic propensity. The c-Ha-ras oncogene alone, or combined with v-myc, transfected into early passage rat embryo fibroblasts, induce these cells to secrete high levels of type IV collagenolytic metalloproteinase and to concomitantly exhibit a high incidence of spontaneous metastases in nude mice. Co-transfection of c-Ha-ras plus the adenovirus type 2 Ela gene yields cells which are highly tumorigenic but nonmetastatic and fail to produce type IV collagenase. Regulation of type IV collagenase levels can occur by at least two mechanisms: 1) transcriptional regulation; 2) secretion of the active versus the latent enzyme. A specific region of the enzyme has been identified which controls the active versus the latent state. Mutations in this region could cause the enzyme to be secreted in a constitutively active state.

Major Findings:

We have found that the ras<sup>H</sup> oncogene (either the viral oncogene from the Harvey sarcoma virus or the T24 bladder carcinoma oncogene) when used to transform NIH-3T3 cells, rat embryo fibroblasts, human bronchial epithelial, or human breast carcinoma, results in both tumorigenic and metastatic cells.

Cotransfection of ras + Ela results in suppression of collagenase IV expression. We have obtained a type IV collagenase clone encoding the 5' upstream sequence of the type IV collagenase gene. The induction of type IV collagenase mRNA in ras-transfected fibroblasts is likely to be mediated by positive or negative trans-acting proteins, each recognizing particular cis elements.

A long-range exonuclease will be employed to define the sites of specific protein DNA interactions in the vicinity of the type IV collagenase promoter. With this assay, fragments of DNA up to several kilobase pairs in length are labeled, incubated with non-specific competitor and extracts of appropriate cells (in this instance ras-plus and ras-minus fibroblasts); the desired complexes are highly enriched by immunologic precipitation of a specifically bound tag, a lac repressor beta galactosidase fusion protein. The recovered complexes will be degraded with T7-gene-6-exonuclease and the digested products displayed by denaturing electrophoresis. If the binding of a nuclear protein is directly modulated by ras, or repressed by Ela, this assay has a high potential of identifying the locus of its action. By transfection of a chimeric gene composed of a reporter, such as chloramphenicol acetyl transferase, fused to type IV collagenase upstream and promoter sequences with specifically ablated binding sites, the identification of a responsive element could be confirmed. The identification of the transcription factors common to the expression of type IV collagenase and other proteins expressed by invasive cells could define central regulators of metastasis.

We have identified an amino acid sequence PRCGVDPV which contains an unpaired cysteine residue and regulates the active versus the latent state of type IV collagenase. A mutation in this sequence (particularly the cysteine) would result in an intrinsically activated enzyme. Furthermore, the mutation would only have to occur on one allele because the active enzyme dominates the latent enzyme. PCR will therefore be used to study the frequency of a mutation in this intrinsic suppressor sequence in human carcinomas during progression.

Publications:

Liotta LA. H-ras and the metastatic phenotype. (Editorial) J Natl Cancer Inst 1988;80:468.

Hoyhtya M, Turpeenniemi-Hujanen T, Stetler-Stevenson W, Krutzsch H, Tryggvason K, Liotta LA. Monoclonal antibodies to type IV collagenase recognize a protein with limited sequence homology to interstitial collagenase and stromelysin. FEBS Lett 1988;233:109-3.

Muschel R, Liotta LA. Role of oncogenes in metastases. *Carcinogenesis* 1988; 9:705-0.

Steeg PS, Bevilacqua G, Pozzatti R, Liotta LA, Sobel ME. Expression of NM23, a gene associated with low tumor metastatic potential, is increased during adenovirus 2 Ela inhibition of experimental metastasis. *Cancer Res* 1988;48: 6550-4.

Liotta LA. Gene products which play a role in cancer invasion and metastasis. 10th San Antonio Breast Cancer Symposium - Plenary Lecture. *Breast Cancer Res Treat* 1988;11:113-24.

Spinucci C, Zucker S, Wieman JM, Lysik RM, Imhof B, Ramamurthy N, Liotta LA, Nagase H. Purification of a gelatin degrading type IV collagenase secreted by ras oncogene transformed fibroblasts. *J Natl Cancer Inst* 1988;80:1416-0.

Stetler-Stevenson WG, Krutzsch HC, Wachter MP, Margulies IMK, Liotta LA. The activation of human type IV collagenase proenzyme. Sequence identification of the major conversion product following organomercurial activation. *J Biol Chem* 1989;264:1353-6.

Liotta LA, Stetler-Stevenson WG. Metalloproteinases and malignant conversion: Does correlation imply causality? (Editorial) *J Natl Cancer Inst* 1989;81: 556-7.

Schiffmann E, Stracke ML, Liotta LA. Metastasis. In: Sporn MB, Roberts AB eds. Peptide growth factors and their receptors. Handbook of experimental pharmacology. Springer-Verlag (in press)

Liotta LA. Oncogene induction of metastases. Ciba Foundation Symposium No. 141, London, England, April 19-21, 1988. New York: John Wiley & Sons Ltd. (in press)

Liotta LA, Stetler-Stevenson W. Principles of molecular cell biology of cancer: Cancer metastasis. In: DeVita VT, Jr, Hellman S, Rosenberg SA, eds. Cancer: principles and practice of oncology, 3rd ed. Philadelphia: J B Lippincott Co. (in press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09130-05 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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:	M.E. Sobel	Senior Investigator	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
	A.P. Claysmith	Biologist	LP NCI
	V. Cioce	Guest Researcher	LP NCI
	V. Castronovo	Breast Cancer Fellow	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:

0.5

## PROFESSIONAL:

0.4

## OTHER:

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.)

Laminin, a major glycoprotein of basement membranes, exhibits saturable binding to the surface of certain neoplastic and normal cells. Examination of various cell types (e.g., human pancreatic carcinoma, melanoma, bladder carcinoma, colon carcinoma) via live cell binding techniques indicates the number of receptors to be 50,000-110,000 per cell depending on cell type. In our laboratory, the laminin receptor has been isolated from human breast carcinoma cells and tissue, and mouse and human 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. With the isolation and characterization of a cDNA clone for the human and murine laminin receptors, we have predicted the complete sequence of the laminin receptor, and have developed anti-laminin receptor synthetic peptide antibodies as well as cDNA restriction fragments which are being used to assess the cell surface expression of the laminin receptor as well as laminin receptor mRNA levels in different neoplastic cell types. The proposed study is designed to measure and correlate laminin receptor cell surface expression and mRNA levels with clinical information. Preliminary studies indicate a significant increase in laminin receptor expression in malignant versus benign breast tissues. These findings suggest a site for possible intervention in the sequence of tumor invasion and metastasis.



### Major Findings:

The goal of this project is to correlate laminin receptor cell surface expression and mRNA levels with clinical information. Clinical data on each case includes estrogen and progesterone receptor content, histologic grade, clinical stage including lymph node status, breast quadrant location of primary tumor and pre-versus post-menopausal status. Laminin receptor expression is evaluated by immunologic and hybridization techniques.

We have concentrated our efforts on obtaining specific antibodies to the human laminin receptor which can be used to assess cell surface laminin receptor expression. We synthesized twelve different synthetic peptides derived from the laminin receptor cDNA clone, and immunized rabbits. The anti-laminin receptor synthetic peptides were affinitypurified and their specificity was tested by indirect ELISA. Using indirect immunoperoxidase methods, we have developed excellent specific staining for the laminin receptor on breast tissue specimens. In addition, indirect immunofluorescence methods have been developed to study isolated breast carcinoma cells. Qualitative assessment of the staining intensity as a means to determine relative amounts of the protein in various tissues and cells revealed intense staining for the laminin receptor in invading breast carcinoma cells. In in vitro model systems, laminin receptor content as measured by these assays was higher in poorly differentiated MDA-MB 231 breast tumor cells which were negative for steroid hormone receptors than in steroid-responsive T47D cells.

In addition, we have determined that in breast carcinomas, the levels of laminin receptor mRNA as assayed by RNA blot hybridization are directly correlated with laminin receptor cell surface expression. For example, the levels of laminin receptor mRNA are higher in steroid hormone-negative MDA-MB 231 breast carcinoma cells than in T47D human breast carcinoma cells which contain estrogen and progesterone receptors. We have initiated a study in which total cellular RNA is extracted from cases of breast carcinoma, and the levels of laminin receptor mRNA are being measured in tumor versus normal breast tissue. We are developing an in situ laminin receptor hybridization assay to determine laminin receptor mRNA levels in individual cells of breast tumors. This method will be particularly applicable for determination of the heterogeneity of receptor expression within the cells of a tumor, as well as in situations where tissue sample is limited in quantity. We have succeeded in subcloning a restriction fragment of the human laminin receptor into a riboprobe vector and have obtained antisense and sense (negative control) probes to measure in situ mRNA levels.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09131-05 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Molecular Cloning of Connective Tissue Matrix Molecules

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

PI:	M.E. Sobel	Senior Investigator	LP NCI
OTHER:	A.P. Claysmith	Biologist	LP NCI
	V. Castronovo	Breast Cancer Fellow	LP NCI
	M. Fernandez	Visiting Fellow	LP NCI
	V. Cioco	Guest Researcher	LP NCI
	H.-S. Chen	Stay-in-School	LP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Tumor Invasion and Metastases Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

3.0

## OTHER:

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 interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. To better understand the protein components that make up the extracellular matrix, their regulation, and how they interact with the tumor cell, we have undertaken to construct, isolate, and characterize molecular clones of laminin and laminin receptor. Laminin is a high molecular weight glycoprotein specifically associated with basement membranes. The laminin receptor is a cell surface protein to which laminin specifically binds with high affinity. We have previously isolated cDNA clones of the human and murine laminin receptor, and have predicted the entire primary structure of the receptor in both species. The nascent laminin receptor predicted from the cloning experiments has an apparent molecular mass of 37 kilodaltons, while the mature laminin receptor on cell surfaces has an apparent molecular mass of 67 kilodaltons. Antibodies to synthetic peptides deduced from the cDNA clones have been used to study the biosynthesis of the receptor. A precursor-product relationship between the cDNA-predicted nascent protein and the mature 67 kilodalton polypeptide was established. The level of laminin receptor mRNA in steroid hormone-responsive breast carcinoma cells was found to be modulated by estrogen and progesterone. Multiple genes for the laminin receptor have been detected in both mouse and human. At least some of these genes are pseudogenes; the transcripts of the pseudogenes are very minor components of the total cellular laminin receptor mRNA. We have established a correlation between total cellular laminin receptor mRNA, translatable laminin receptor mRNA, and the number of cell surface laminin receptors in breast carcinoma cells.

### Major Findings:

Regulated expression of the laminin receptor. The effects of estradiol and two synthetic progestins on the expression of the laminin receptor in two human breast carcinoma cell lines were examined. The T47D cell line contains estrogen and progesterone receptors, while the MDA-MB 231 cell line lacks both of these receptors. Treatment of T47D cells with estradiol alone resulted in a several-fold increase in the steady-state level of laminin receptor mRNA as well as in cell surface laminin receptor expression. A more dramatic increase of mRNA and cell surface expression in T47D cells was observed after treatment with estradiol plus progestin, or with progestin alone. Estradiol treatment also increased chemotaxis and haptotaxis of T47D cells to laminin, but had no effect on the attachment of these cells to laminin. Progesterone treatment had the converse effect on chemotaxis and attachment of T47D cells. These results suggest that the various cell-laminin interactions are mediated by different mechanisms, and that laminin receptor levels are a necessary but not the sole determinant of laminin-related cell behavior. There were no effects of estradiol or progesterone on the receptor-negative MDA-MB 231 cells, however the latter cells had greater levels of laminin receptor mRNA under all tested conditions than did the T47D cells. The augmentation of laminin receptor mRNA by estrogen and progesterone treatment in hormone receptor-positive cells, but not in cells that lack these receptors, and the increased levels of laminin receptor mRNA in the steroid receptor-negative cells under all tested conditions, may relate functionally to the difference in the clinical aggressiveness between classes of breast cancers.

Anti-laminin receptor synthetic peptide antibodies. The complete cDNA sequence of the laminin receptor clone was determined. Computer analysis predicted a putative, short transmembrane domain, with the amino terminus of the protein in the cytoplasmic space, and the carboxy-terminal two thirds of the receptor in the extracellular domain. Twelve different regions of the cDNA-predicted sequence were selected, and synthetic peptides were synthesized. Rabbits were immunized with the synthetic peptides, and antibodies were affinity-purified. These antibodies are being used in a variety of studies, including mapping cytoplasmic versus extracellular domains of the receptor by immunofluorescence, immunoprecipitation of the receptor from cells, determination of the biosynthesis of the receptor (see below), as well as the development of an ELISA for determination of laminin receptor in cells.

Biosynthesis of the laminin receptor. A full-length cDNA clone of the laminin receptor predicts a molecular mass of 33 kilodaltons. The nascent protein synthesized by a rabbit reticulocyte cell-free translation system from selectively-hybridized laminin receptor mRNA has an apparent molecular mass on SDS-polyacrylamide gels of 37 kilodaltons. The relationship between the 37 kilodalton nascent protein and the mature 67 kilodalton cell surface laminin receptor was studied using affinity-purified antibodies directed against synthetic peptides predicted by the laminin receptor cDNA sequence. On immunoblots of human melanoma cell extracts, antibodies to specific domains of the receptor recognize either a 67 kilodalton polypeptide, a 37 kilodalton polypeptide, or both proteins. In [<sup>35</sup>S] methionine pulse-chase experiments, anti-laminin receptor synthetic peptide antibodies immunoprecipitated a 37 kilodalton protein which was chased into a 67 kilodalton polypeptide within 30 minutes. These



results demonstrate that the 37 kilodalton nascent protein predicted by the laminin receptor cDNA is the precursor for the 67 kilodalton cell surface metastasis-associated laminin receptor.

Translatable levels of laminin receptor mRNA. We have previously determined that laminin receptor mRNA in the tumor cell is a rate-limiting control step in the biosynthesis of the receptor, and hence in the regulation of cellular attachment to basement membranes via laminin. We have extended these findings by correlating the total cellular laminin receptor mRNA (as determined by RNA blot hybridization analysis) with the amount of translatable laminin receptor mRNA in human breast carcinoma cells. Total cellular RNAs from two subclones of the metastatic MCF7 breast carcinoma cell line, one with high levels of laminin receptor mRNA, and one with low levels of the receptor, were selectively-hybridized to laminin receptor cDNA restriction fragments, and the pure hybridized laminin receptor mRNA was assayed in a cell-free translation system. The amount of translated laminin receptor nascent (37 kilodalton) protein from each cell line correlated with the relative amounts of hybridizable laminin receptor on RNA blots, as well as with immunofluorescence staining of the laminin receptor on the cell surfaces and with Scatchard analysis of the laminin binding sites on these cells. These studies suggest that in breast carcinomas, the amount of hybridizable laminin receptor mRNA is an excellent indicator of the total expression of cell surface laminin receptor.

Multiple genes for the human laminin receptor. Using genomic DNA hybridization blots, we have previously predicted that there are multiple laminin receptor genes in the human and mouse genomes. Several cDNA clones have been isolated by cross-hybridization which have alternate sequences. These so-called pseudo-gene transcripts are being completely sequenced. They may contain unspliced intron sequences due to mutations at the joints of the intron-exon boundaries in the pseudogene DNA sequence. To assess the contribution of pseudogene transcripts to the total cellular hybridizable laminin receptor mRNA, we developed synthetic oligonucleotides specific for pseudo versus authentic laminin receptor mRNAs, and assessed the relative levels of pseudo and authentic laminin receptor mRNAs on RNA blots. The pseudo mRNA sequences are undetectable, suggesting that they are a very minor component of the total cellular hybridizable mRNA. This is consistent with our other findings that cellular levels of laminin receptor mRNA directly correlate with cell surface expression of laminin receptor in breast carcinoma cells, and validates the RNA blot technique for assessment of laminin receptor differential expression.

#### Publications:

Gastronovo V, Taraboletti G, Liotta LA, Sobel ME. Modulation of laminin receptor expression by estrogen and progestins in human breast cancer cell lines. *J Natl Cancer Inst* 1989;81:781-8.

Rao CN, Gastronovo V, Schmitt MC, Wewer UM, Claysmith AP, Liotta LA, Sobel ME Evidence for a precursor of the high affinity metastasis-associated murine laminin receptor. *Biochemistry* (in press)



Sobel ME, Liotta LA. Receptor-ligand interactions: Role in cancer invasion and metastasis. In: Vogel H, ed. P & S Biomedical Sciences Symposia "Molecular Mechanisms in Cellular Growth and Differentiation". (in press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09163-02 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Anticancer Effects of a Novel Drug, Merck L651582

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

PI:	E.C. Kohn	Medical Staff Fellow	MB NCI
OTHER:	E. Schiffmann	Research Chemist	LP NCI
	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI

## COOPERATING UNITS (if any)

C. Myers, Chief, Med. & Clin. Pharmacol. Branch, NCI; D. Hupe, Merck Res. Labs., Rahway, NJ; M.A. Sandeen, Research Technician, FCRF/DCBD Animal Holding Unit

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Tumor Invasion and Metastases Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.3

## OTHER:

0.7

## CHECK APPROPRIATE BOX(ES)

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

D

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

We have continued our investigation on the effect of MSD L651582, a potential novel anti-cancer drug. We have shown previously that L651582 reversibly inhibits stimulated tumor cell motility and adhesion, inhibits autocrine motility factor-stimulated phosphatidyl inositol metabolism, and decreases spontaneous pulmonary metastases in experimental animal models. We have expanded our in vivo observations to include other tumor cell types and methods of administration. Daily oral and intraperitoneal (IP) administration of drug can be done successfully as well as use of continuous subcutaneous treatment using Alzet mini-osmotic pumps. Nude mice with heavy tumor burdens of OVCAR3 human ovarian cancer had marked prolongation of life expectancy with once daily IP administration of L651582. Mice bearing L1210 murine leukemia had improvement in overall survival when treated with the combination of L651582, and cyclophosphamide at subtherapeutic doses. These animal observations suggest an important anti-proliferative role for L651582 as a new treatment for cancer. These data and projected phase I clinical studies will be presented to Merck Research Laboratories for initiation of an IND (investigational new drug approval). The above uses have been summarized as part of a use patent application.

Major Findings:

We have been studying a novel anti-cancer agent developed by Merck Research Laboratories, L651582. This compound indirectly inhibits several steps in de novo and salvage nucleotide biosynthesis. It also indirectly inhibits arachidonic acid metabolism, phosphatidyl inositol metabolism and its secondary calcium signal. L651582 has been shown to inhibit keratinocyte proliferation in vitro and in vivo in a pig skin psoriasis model.

We have previously shown that this agent 1) decreases autocrine motility factor (AMF) and laminin-stimulated tumor cell motility; 2) inhibits tumor cell adhesion to tissue culture plastic, laminin and fibronectin substrata; 3) decreases AMF-stimulated phosphatidyl inositol metabolism; and 4) inhibits tumor cell proliferation in vitro.

Over the course of the last year, we have been investigating the effects of L651582 in animal models of tumorigenesis and metastasis. We have shown that daily oral administration of this agent to nude mice is feasible and tolerable. We have studied the efficacy of orally administered L651582 against L1210 bearing nude mice. Mice receiving doses of 500 mg/kg/d had overall survival of 110 - 140% of mice receiving methylcellulose control solution. L651582 given orally in combination with oral daily cyclophosphamide (40 mg/kg/d) prolonged survival up to 160% of control animals, and approximately 130% of those mice receiving cyclophosphamide alone. Intraperitoneal experiments using a log lower concentration produced a similar profile of results. Studies are in progress to evaluate the efficacy of continuous infusion of L651582 via Alzet pumps in these leukemia-bearing nude mice.

Most recently, we have seen striking results with intraperitoneally administered L651582 to OVCAR3 human ovarian carcinoma-bearing nude mice. Eight mice matched for degree of malignant ascites were split into two groups for DMSO control v. L651582, 50 mg/kg/d in 60% DMSO given daily. The mean survival from the start of treatment for the control group was 7.4 +/- 4 days, whereas the treated group was 16.0 +/- 2.5 days, representing a prolongation of life of 220%. A repeat study with 12 animals per arm is ongoing now. An invention report has been filed and a use patent is being written presently.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09164-02 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Role of Collagenolytic Metalloproteinases in Metastases

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

PI:	W.G. Stetler-Stevenson	Senior Staff Fellow	LP NCI
OTHER:	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
	M. Wachter	Visiting Scientist	LP NCI
	H.C. Krutzsch	Expert Consultant	LP NCI
	A. Levy	Microbiologist	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:

3.5

## PROFESSIONAL:

2.5

## OTHER:

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.)

In order to investigate the role of type IV collagenase in tumor invasion and metastases, we have undertaken the purification of this enzyme, studied the Michaelis-Menten kinetics of this enzyme, isolated a full-length cDNA clone and characterized specific structure-function relationships using primary sequence information. Our study of the mechanism of an *in vitro* activation of type IV collagenase revealed a region in the proenzyme fragment, adjacent to the activation locus that is highly conserved amongst the neutral metalloproteinases studied so far. Studies using synthetic peptide analogs of this domain showed that it contains a unpaired cysteine residue that functions to maintain the type IV procollagenase in a latent form, and under conditions of substrate saturation, determined from kinetic analyses, acts as a competitive inhibitor of this enzyme. We have also developed an assay to measure the binding of type IV collagenase to both type IV collagen and gelatin. These studies demonstrate that enzyme binding to these substrates is independent of metal ion binding and can be blocked by fibronectin and fibronectin gelatin-binding domain peptides. Using synthetic oligonucleotides as probes, we have obtained a full-length 3.1 kB cDNA clone for type IV collagenase, and two partial cDNA clones of 2.8 and 1.0 kB both of which contain the 5' untranslated portion of the message. Using these probes, we are currently exploring the levels of type IV collagenase mRNA in various human tumor samples and attempting to correlate these data with tumor stage and patient survival. These probes are also being used to screen a human genomic DNA library to obtain a clone for the complete type IV collagenase gene. These clones will be used to study the regulation of type IV collagenase gene expression.



Major Findings:

Type IV collagenase is a neutral matrix metalloproteinase that degrades type IV collagen within the triple helical domain. This enzyme is secreted as a latent 72 kDa protein by human A2058 melanoma cells in culture. *In vitro* assays have shown that highly purified preparations of latent enzyme undergo concomitant autoproteolysis with attainment of full collagenolytic activity, following treatment with organomercurial compounds. This autocatalyzed reaction results in the generation of a new amino terminus with the sequence YNFFPRKPKWKDNQ. The proenzyme fragment that is lost contains 80 amino acid residues. This proenzyme fragment contains a region in its carboxy terminal end, immediately adjacent to the site of cleavage, with the sequence PRCGVPDV which is highly conserved amongst all members of the matrix metalloproteinase family that have been studied. This sequence also contains an unpaired cysteine residue. Synthetic peptide analogs of this sequence can inhibit the activated enzyme suggesting that in the native latent enzyme, this region may act as an endogenous inhibitor and function to maintain the proenzyme in an inactive form.

The binding of type IV collagenase to type IV collagen, type I collagen and gelatin was studied by developing a solid phase binding assay. In this assay the binding phase, collagen or gelatin, is immobilized on an inert substrate. The binding of  $^{125}\text{I}$ -type IV collagenase can then be measured directly. This assay was used to measure the binding affinity for these substrates as well as other matrix macromolecules to compete with type IV collagenase binding. Type IV collagenase binding to type IV collagen or gelatin is independent of metal ion binding and can be blocked by fibronectin and fibronectin gelatin-binding domain peptides. This confirms the similarity in the binding domain of type IV collagenase and fibronectin for type IV collagen and gelatin. Type IV collagenase is a metalloproteinase that is readily inhibited by metal ion chelating agents such as EDTA. The putative metal binding domain was identified using synthetic peptide analogs. Synthetic peptides with the sequence VAAHEFGHAMGLEHS functioned as inhibitors of the gelatin zymogram activity of this enzyme. We have used this system to explore the function of the histidine residues in this sequence and found that two of the three are required to maintain inhibitory activity.

Synthetic oligonucleotide probes based on the amino terminal portion of type IV collagenase were synthesized and used to probe a placental cDNA library in the lambda gt11 vector. A positive clone of 1.0 kB was obtained on the initial screening. Sequencing of this clone demonstrated that it corresponds to the 5' end of the message and contains the previously unreported 5' untranslated region and signal peptide sequences. This clone was used to rescreen the library. On rescreening, a 2.8 kB and presumably full-length 3.1 kB clones were obtained and subsequently sequenced. These clones extended in the 3' direction from the 1.0 kB clone previously described. These cDNA clones were used to study the levels of type IV collagenase expression in various tumor cell lines as well as in actual human tumor samples, and to screen a genomic library to obtain the complete type IV collagenase gene for future studies.

Publications:

Hoyhtya M, Turpeenniemi-Hujanen T, Stetler-Stevenson W, Kruttsch H, Tryggvason K, Liotta L. Monoclonal antibodies to type IV collagenase recognize a protein containing limited sequence homology with interstitial collagenase stromelysin. FEBS Lett 1988;233:109-3.

Liotta LA, Stetler-Stevenson WG. Principles of molecular cell biology of cancer: cancer metastasis. In: DeVita VT, Jr., Hellman, S, Rosenberg SA (eds.): Cancer: principles and practice of oncology, 3rd ed. Philadelphia: J B Lippincott Co.

Stetler-Stevenson WG, Kruttsch H, Wachter M, Margulies IMK, Liotta LA. The activation of human type IV collagenase proenzyme. J Biol Chem 1989;264:1353-6.

Liotta LA, Stracke ML, Wewer UM. Tumor invasion and metastases: Biochemical mechanisms. In: Magrath I, ed. New directions in cancer treatment. Springer-Verlag (in press)

Liotta LA, Stracke ML, Wewer UM, Schiffmann E. Tumor invasion and metastases: Biochemical mechanisms. In: The pathobiology of neoplasia. Plenum Press (in press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09179-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Novel Metalloproteinase Inhibitors: Role in Tumor Invasion and Metastasis

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

PI: W.G. Stetler-Stevenson

Senior Staff Fellow

LP NCI

OTHER: H. Krutzsch

Expert Consultant

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:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Human melanoma cells secrete a 21 kDa protein, termed CSC-21K, which binds with 1:1 molar stoichiometry to the matrix metalloproteinase type IV collagenase proenzyme secreted by the same cells. This binding protein has been purified and its primary structure determined. The amino acid sequence demonstrates that this protein shares significant homology with human TIMP (tissue inhibitor of metalloproteinase), including conservation of the positions of the twelve cysteine residues. The identification of CSC-21K now indicates that a family of TIMP-related proteins exists. Individual members of this family may possess selective affinities for different members of the matrix metalloproteinase family. CSC-21K produced by tumor cells can also be considered as an onco-suppressor gene product, because it could play an important role in regulating the metalloproteinases involved in tumor invasion and angiogenesis.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09180-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Identification of Novel Metastasis Associated Gene Products

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

PI:	W.G. Stetler-Stevenson	Senior Staff Fellow	LP NCI
OTHER:	E. Kohn	Staff Fellow	MB NCI
	L.A. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Tumor Invasion and Metastases Section

## INSTITUTE AND LOCATION

NCI, NTH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## 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.)

We have used synthetic oligonucleotide probes to screen a human melanoma cDNA library. Probes containing sequences similar to those contained in metastasis-associated metalloproteinases were used to screen this library. Screening yielded several cDNA clones. The largest containing a 1.7 kB insert was selected for further characterization. Sequencing of this clone revealed that it contained a novel sequence with a single open reading frame. Translation of the cDNA sequence to obtain the deduced amino acid sequence yielded a unique protein sequence that contained an initiating methionine followed by a signal peptide sequence. The 3' end of this clone contains a polyadenylation signal site and poly A tail. Computerized homology searches revealed no homology with known protein sequences. Synthetic peptides corresponding to the deduced amino acid sequence were prepared and used to prepare antipeptide antibodies. Western blot analysis of conditioned media from a variety of tumor cell lines demonstrated reactivity with a secreted protein of approximately 44 kDa, as well as some lower molecular weight material. The significance of these results and the possibility of using this as a tumor cell marker is being explored.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00550-09 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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	BRMP NCI
	M. Raffeld	Senior Staff Fellow	LP NCI
	M.A. Bookman	Senior Investigator	MB NCI
	M.A. Stetler-Stevenson	Expert	LP NCI
	P. Lardelli	Guest Researcher	LP NCI
	L. Jeffrey Medeiros	Senior Staff Fellow	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:

4.0

## PROFESSIONAL:

3.0

## OTHER:

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.)

In order to assess the clinical and pathologic significance of the immunologic characterization of human malignant lymphomas, fresh biopsy tissues are obtained from patients referred to the Clinical Center for treatment. Biopsies are obtained with patient permission prior to therapy and processed in the Hematopathology Section. The neoplastic cells are characterized as to their origin from T cells, B cells, or histiocytes, and in addition can be identified as belonging to specific developmental and functional subpopulations. This data is then correlated with clinical and pathologic data. Morphologic features are analyzed to achieve improved classification of lymphoproliferative lesions.

This information is utilized to develop improved classifications of disease and to distinguish new clinicopathologic entities. It also will be used as a basis for potential immunotherapy or adjunctive immunotherapy in a program of autologous bone marrow transplantation.

Major Findings:

A concept for the classification of histiocytic disorders was developed. Reactive and neoplastic proliferations of histiocytes can be related to the two main subpopulations of histiocytic cells: phagocytic histiocytes or macrophages and dendritic cells. Malignant histiocytic proliferations are rare. Most true histiocytic lymphomas present as tumor masses, often with stage IV disease at presentation, with frequent involvement of bone and skin. Systemic histiocytic proliferations, i.e., malignant histiocytosis, if they exist, are closely related to acute monocytic leukemia and represent differing clinical presentations of the same neoplastic process. Many conditions formerly diagnosed as "malignant histiocytosis" represent either hemophagocytic syndromes or large cell immunoblastic lymphomas with sinusoidal involvement.

A study of proliferative lesions composed of dendritic cells (interdigitating reticulum cells and dendritic reticulum cells) is underway with analysis of immunophenotypic, clinical and pathologic features.

An analysis of the histologic category of diffuse, mixed small and large cell lymphomas has been undertaken using immunophenotypic and molecular genetic approaches. Two subgroups have been identified: 1) peripheral T-cell lymphomas and 2) "B-cell lymphomas with T-cell predominance" or "pseudo-peripheral T-cell lymphomas". Correlations will be drawn with clinical and pathologic features.

A clinicopathologic and phenotypic study of lymphocytic lymphoma of "intermediate" differentiation (IDL) was completed. The nuclear antigen K1-67 was shown to be a useful parameter in correlating with prognosis and was more predictive than mitotic rate in identifying patients with a poor prognosis. A blastic or accelerated form of the disease was also identified and characterized histologically. Clinically, IDL resembles a low-grade, non-Hodgkin's lymphoma in that there is a continuous relapse rate over time with no patient having a prolonged disease-free survival.

Publications:

Jaffe ES, Lipford EH Jr, Margolick JB, Longo DL, Cossman J, Fauci AS. Lymphomatoid granulomatosis and angiocentric lymphoma: A spectrum of post-thymic T-cell proliferations. *Sem Resp Med* 1989;10:167-2.

Sundeen JT, Cossman J, Jaffe ES. Lymphocyte predominant Hodgkin's disease nodular subtype with coexistent "large cell lymphoma": Histological progression or composite malignancy? *Am J Surg Pathol* 1988;12:599-6.

Steinberg A, Seldin M, Jaffe ES, Smith H, Klinman D, Krieg A, Cossman J. Angioimmunoblastic lymphadenopathy with dysproteinemia. *Ann Intern Med* 1988; 108:575-4.

Solomon D, Merino MJ, Linehan M, Jaffe ES. Angiocentric malignant lymphoma involving lung and kidney: mimicking renal cell carcinoma in fine needle aspiration cytology. *Surg Pathol* 1988;1:273-6.

Publications (Cont'd):

- Jaffe ES. Histiocytoses of lymph nodes: Biology and differential diagnosis. *Semin Diag Pathol* 1988;5:376-90.
- Barriga F, Whang-Peng J, Lee E, Morrow C, Jaffe ES, Cossman J, Magrath IT. Development of a second clonally discrete Burkitt's lymphoma in a human immunodeficiency virus (HIV) positive homosexual patient. *Blood* 1988;72:792-5.
- Jaffe ES. Immune markers in the diagnosis of malignant lymphomas. *J Virol Meth* 1988;21:265-4.
- Young RC, Longo DL, Glatstein E, Ihde DC, Jaffe ES, DeVita VT, Jr. The treatment of indolent lymphomas: Watchful waiting versus aggressive combined modality treatment. *Semin Hematol* 1988;25:11-6.
- Rosolen A, Nakanishi M, Poplack DG, Cole D, Quinones R, Reaman G, Cotelingam JD, Trepel JB, Sausville E, Marti GE, Jaffe ES, Neckers LM, Colamonici OR. Expression of interleukin-2 receptor  $\beta$  subunit in hematopoietic malignancies. *Blood* (in press)
- Ladisch S, Jaffe ES. The histiocytoses. In: Pizzo PA, Poplack DG, eds. *Principles and practice of pediatric oncology*. Philadelphia: J B Lippincott Co, 1988;477-90.
- Lipford EH, Margolick JB, Longo DL, Fauci AS, Jaffe ES. Angiocentric immunoproliferative lesions: A clinicopathologic spectrum of post-thymic T-cell proliferations. *Blood* 1988;72:1674-1.
- Surbone A, Longo DL, DeVita VT, Jr, Ihde DC, Duffey PL, Jaffe ES, Solomon D, Hubbard SM, Young RC. Residual abdominal masses in aggressive non-Hodgkin's lymphoma after combination chemotherapy: significance and management. *J Clin Oncol* 1988;6:1832-7.
- Haddy TB, Keenan AM, Jaffe ES, Magrath IT. Bone involvement in young patients with non-Hodgkin's lymphoma: efficacy of chemotherapy without local radiotherapy. *Blood* 1988;72:1141-7.
- Jaffe ES. Lymph nodes. Reactive and neoplastic conditions. In: Zucker-Franklin D, Greaves MF, Grossi CE, Marmont AM, eds. *Atlas of blood cells - function and pathology*, 2nd ed. Philadelphia: Lea & Febiger, 1988;549-98.
- Wagner DK, Marti GE, Jaffe ES, Straus SE, Nelson DL, Fleisher TA. Lymphocyte analysis in a patient with X-linked agammaglobulinemia and isolated growth hormone deficiency after development of echovirus dermatomyositis and meningoencephalitis. *Int Arch Allerg Appl Immunol* (in press)
- Folks TM, Kessler SW, Orenstein JM, Justement J, Jaffe ES, Fauci AS. Infection and replication of HIV-1 in purified progenitor cells of normal human bone marrow. *Science* 1988;242:919-2.

Publications (Cont'd):

Giardina SL, Young HA, Faltynek CR, Jaffe ES, Clark JW, Steis RG, Urba WJ, Mathieson BJ, Gralnick H, Lawrence J, Overton WR, Longo DL. Rearrangement of both immunoglobulin and T-cell receptor genes in a prolymphocytic variant of hairy cell leukemia patient resistant to interferon-alpha. Blood 1988;72:1708-6.

Jaffe ES, Ulirsch RC. Salivary gland lymphadenopathies associated with AIDS: reply. (Letter to the Editor) Hum Pathol 1988;19:1120.

Elias JM, Gown AM, Nakamura RM, Wilbur DC, Herman GE, Jaffe ES, Battifora H, Brigati DJ. Quality control in immunohistochemistry - Report of a workshop sponsored by the Biological Stain Commission. Am J Clin Pathol (in press)

Jaffe ES. The elusive Reed-Sternberg cell. (Editorial) N Engl J Med 1989; 320:529-1.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00552-09 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Molecular Basis of the Diagnosis of Human Lymphoproliferative Disease

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

PI:	J. Cossman	Senior Investigator	LP NCI
OTHER:	M. Raffeld	Senior Staff Fellow	LP NCI
	M. Stetler-Stevenson	Expert	LP NCI
	J. Medeiros	Biotechnology Fellow	LP NCI
	L. Elwood	Senior Staff Fellow	LP NCI
	D. Cohen	Senior Investigator	LCP NIDDKD
	N. Harris	Professor	Harvard University

## COOPERATING UNITS (if any)

Medicine Branch, NCI; Metabolism Branch, NCI

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Hematopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.5

## OTHER:

.50

## CHECK APPROPRIATE BOX(ES)

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

B

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

We have designed and reported a highly sensitive technique to detect minimal disease in follicular lymphoma using PCR. In a prospective study, peripheral blood samples obtained from several hundred lymphoma patients are being analyzed for the presence of circulating t(14;18) lymphoma cells. We have undertaken this longitudinal clinical trial to determine whether relapse can be predicted early using PCR.

To expand the applicability of PCR to the diagnosis of lymphoma, we are (1) developing oligonucleotide primers specific for each of the four breakpoint regions so that nearly all (approximately 90% of t(14;18) lymphomas can be detected, (2) designing PCR amplification from paraffin-embedded fixed-tissue and (3) investigating bcl-2 expression in tissue sections using our anti-bcl-2 antiserum and monoclonals to the bcl-2 protein now under development.

We have shown that the T cell receptor delta chain gene is frequently rearranged in both T and pre-B ALL. By contrast, in most mature T cell neoplasms, we found that both T- $\delta$  alleles have been deleted from the genome. We are now investigating the manner whereby T $\delta$  rearrangement and deletion occurs during T cell development by analyzing these events in our  $\alpha/\beta$  T cell neoplasms.

To determine whether the sensitive detection of clonality by gene rearrangement analysis is useful in differential diagnosis and predicts a malignant outcome, we have selected a specific anatomic site, the orbit, prone to be involved by lymphocytic infiltrates whose malignant behavior is difficult to predict by morphology alone. Among 20 such patients gene rearrangement analysis proved to be more sensitive than immunophenotyping for detecting B cell clones. Clinical follow-up, rebiopsy and clonal analysis demonstrated that gene rearrangements can predict a malignant outcome.

Major Findings:

1. PCR can be used to detect occult disease in follicular lymphoma.
2. The T- $\delta$  gene is deleted from the genome of most mature  $\alpha/\beta$  T cell neoplasms.
3. Gene rearrangements are more sensitive than either conventional morphology or immunophenotyping in detecting clones and predicting malignant behavior in lymphoproliferative processes of the orbit.

Publications:

Uppenkamp M, Andrade R, Sundeen J, Raffeld M, Coupland R, Cossman J. Diagnostic interpretation of Ty gene rearrangement: Effect of polyclonal T cells. Hematolog Pathol 1988;2:15-4.

Steinberg AD, Seldin MF, Jaffe ES, Smith HR, Klinman DM, Krieg AM, Cossman J. Angioimmunoblastic lymphadenopathy with dysproteinemia. Ann Intern Med 1988; 108:575-4.

Stetler-Stevenson MA, Raffeld M, Cohen P, Cossman J. Detection of occult follicular lymphoma by specific DNA amplification. Blood 1988;72:1822-5.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00850-07 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Clonal Analysis of Lymphoid Neoplasia

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

PI:	J. Cossman	Senior Investigator	LP NCI
OTHER:	M. Raffeld	Senior Staff Fellow	LP NCI
	D. Cohen	Senior Investigator	LCP NIDDKD
	M. Stetler-Stevenson	Expert	LP NCI
	C. Hua	Guest Researcher	LP NCI
	A. Pullman	Senior Staff Fellow	LCP NIDDKD

## COOPERATING UNITS (if any)

Laboratory of Clinical Biology, NIDDKD

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Hematopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.75

## PROFESSIONAL:

1.5

## OTHER:

.25

## CHECK APPROPRIATE BOX(ES)

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

B

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

We have cloned and sequenced a t(14;18) bcl-2 breakpoint cluster region which lies more than 12 kb 3' of the major breakpoint cluster region (mbr, located in the last exon of bcl-2), and 5' of the minor cluster region (mcr). Using probes of these separate regions, we have determined the frequency with which each of the breakpoint areas of bcl-2 is involved in translocation and are investigating whether the specific breakpoint of bcl-2 might influence clinical behavior of lymphomas.

To determine whether point mutation in the bcl-2 ORF might be involved in de-regulation of bcl-2, we have directly sequenced PCR-amplified DNA from eleven primary follicular lymphomas. As no mutations were found, we conclude that bcl-2 activation is not generally attributable to mutation in these sites.

In continuing studies of the molecular biology of Hodgkin's disease, we applied PCR to the involved tissues of Hodgkin's disease and found positive bcl-2/J<sub>H</sub> sequences in 33%. Since our single bcl-2/mbr primer detects only 37% of follicular lymphoma, then many, perhaps most, cases of Hodgkin's disease contain t(14;18). Planned studies include investigation of bcl-2 expression using PCR-amplified cDNA and direct analysis of Reed-Sternberg cells for bcl-2 expression in intact tissue sections with an antibody we have raised to the bcl-2 protein.

We have identified a novel T-delta gene rearrangement occurring in the majority of precursor B ("common") ALL. To determine the mechanism of this rearrangement and its consequences, we are cloning and sequencing genomic DNA from a primary case of precursor B ALL which carries the delta rearrangement.

Major Findings:

1. The bcl-2 gene is involved in many, if not most, Hodgkin's lymphomas as demonstrated by PCR amplification of the t(14;18) chromosomal translocation.
2. A consistent, novel T- $\delta$  gene rearrangement occurs in precursor B ALL.

Publications:

Cossman J, Sundeen J, Uppenkamp M, Sussman E, Wahl, L, Coupland R, Lipford E, Raffeld M. Rearranging antigen receptor genes in enriched Reed-Sternberg cell fractions of Hodgkin's disease. Hematol Oncol 1988;6:205-1.

Stetler-Stevenson MA, Raffeld M, Cohen P, Cossman J. Detection of occult follicular lymphoma by specific DNA amplification. Blood 1988;72:1822-1825.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00855-07 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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	LTCC NCI
	P. Levine	Senior Investigator	EEB NCI
	M.A. Stetler-Stevenson	Expert	LP NCI
	L. Al-Gwaiz	Special Volunteer	

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Hematopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.20

## PROFESSIONAL:

0.15

## OTHER:

0.05

## 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.

For cases in which fresh material is not available, DNA will be extracted from paraffin sections and examined for HTLV-I sequences using the PCR amplification technique. This information will be correlated with serologic, clinical and pathologic data to determine the validity of the PCR technique in establishing the diagnosis of adult T-cell lymphoma/leukemia (ATL).

In selected populations where HTLV-I is endemic, such as Jamaica or Trinidad, prospective studies of all newly diagnosed lymphoma patients are conducted. Such studies are useful in identifying the clinicopathologic spectrum of HTLV-I associated diseases. Prospective studies of all lymphomas in similar geographic regions with differing incidences of adult T cell leukemia/lymphomas are included to discern factors which may have an impact on the incidence of HTLV-I and HTLV-I associated diseases.

Major Findings:

A registry for ATL diagnosed in the United States was established. Approximately 100 cases have been entered in the registry thus far; epidemiologic, clinical, and pathologic data are being analyzed.

A prospective study of all malignant lymphomas diagnosed in Trinidad-Tobago over a three-year period has been undertaken. Preliminary studies indicate a high incidence of ATL. It has been possible to demonstrate a T-cell phenotype in paraffin-section immunoperoxidase studies.

In a separate study, serum samples were obtained over a 2 year period from 136 Panamanian patients with hematologic malignancies. Only 3 patients had clinical and serologic findings of HTLV-I-associated ATL. It was concluded that although classical ATL does occur in the Panamanian population, it is not as prevalent as in other Caribbean populations.

Publications:

Levine PH, Jaffe ES, Manns A, Murphy EL, Clark J, Blattner WA. Human T-cell lymphotropic virus type I and adult T-cell leukemia/lymphoma outside of Japan and the Caribbean basin. *Yale J Biol Med* 1988;61:215-2.

Levine PH, Cuevas M, Arosemena JR, Jaffe ES, Saxinger WC, Altafulla M, De Bernal J, Espino H, Rios B, Xatruck H, Barnett M, Drummond J, Alexander S, Blattner, W, Reeves W. Human T-cell leukemia virus (HTLV)-I and hematologic malignancies in Panama. *Cancer* (in press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09181-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Expression of Bcl-2 in Lymphoproliferative Disorders

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

PI: M. Stetler-Stevenson

Expert

LP NCI

## COOPERATING UNITS (if any)

Medicine Branch, NCI

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Hematopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

A

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Follicular lymphoma is a common B-cell neoplasm which has remained largely an incurable disease. Approximately 95% of follicular lymphomas carry the t(14;18) (q32;q21) chromosomal translocation which results in joining of the putative oncogene bcl-2 on chromosome 18 to the immunoglobulin heavy chain joining region gene JH on chromosome 14. Although the deregulation of bcl-2 by the translocation has been proposed as a possible mechanism in the development of neoplasia, the expression of the chimeric bcl-2/JH transcript in follicular lymphoma has not been well studied. We have reverse transcribed RNA extracted from SU-DHL-6 (a cell line containing t(14;18)) and tissues from follicular lymphoma patients and subjected the cDNA to the polymerase chain reaction technique. We were able to detect the chimeric bcl-2/JH cDNA and are quantifying levels of this transcript. Correlation of the clinical course with the level of expression of the chimeric bcl-2/JH transcript expression will indicate the prognostic significance of the proposed bcl-2 deregulation in follicular lymphoma. This will help to illuminate the role of bcl-2 in malignancy and provide a diagnostic test of prognostic value that will assist in patient management. We have previously demonstrated the t(14;18) translocation to be present in 32% of Hodgkin's lymphomas. However, Hodgkin's disease and follicular lymphoma are two distinct clinical-pathological entities. We shall also examine the expression of the bcl-2/JH transcript in Hodgkin's disease patients and again correlate this information with the clinical course. This data will provide further information concerning the role of bcl-2 in hematopoietic malignancies.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09182-01 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.)

Molecular Biology of Human Lymphoproliferative Diseases

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

PI:	M. Raffeld	Senior Staff Fellow	LP NCI
OTHER:	J. Cossman	Senior Investigator	LP NCI
	M.A. Stetler-Stevenson	Expert	LP NCI
	A. Ginsberg	Medical Staff Fellow	LP NCI
	S. Abbondanzo	BTP Fellow	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:

1.3

## PROFESSIONAL:

1.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

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

A, B

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

We have determined the frequency with which each of the breakpoint areas of bcl-2 are involved in translocation and are currently investigating whether the specific breakpoints might influence the clinical behavior of lymphoma. In the course of these investigations, we have cloned and sequenced a t(14:18) bcl-2 breakpoint cluster region which lies more than 12 kb 3' of the major breakpoint cluster region (mbr), and 5' of the minor cluster region (mcr).

We have adapted sensitive PCR technology to the diagnosis of t(14:18) translocated lymphomas. We are currently developing oligonucleotide primers specific for each of the four reported breakpoint clusters so that nearly all t(14:18) translocated lymphomas can be identified. This technology will allow us to follow prospectively large numbers of patients with this lymphoma for both response to treatment and relapse utilizing blood and/or lymph node samples. We are also designing retrospective studies using paraffin embedded tissues.

We have studied a series of recurrent follicular lymphomas to assess the status of their immunoglobulin (Ig) and bcl-2 restriction patterns. We have found that Ig gene restriction pattern changes occur frequently over time (30% of the cases studied). Changes in the bcl-2 gene restriction fragments were not observed. In order for a mutation in an Ig gene to become detectable as a restriction fragment change, we presume that a second unrelated genetic event must have occurred in the variant cell, imparting a selective growth advantage to that cell's progeny. Thus, changes in Ig restriction patterns in the course of a patient's lymphoma, may serve as a marker for the acquisition of secondary growth promoting mutations. We have begun to investigate potential second events which could impart a selective growth advantage to a lymphoma cell or result in aggressive transformation of a lymphoma. To this end, we are currently analyzing the potential role of several oncogenes and anti-oncogenes.



Major Findings:

1. PCR technology can be adapted to the diagnosis of follicular lymphomas with the t(14:18) translocation.
2. Follicular lymphomas show frequent changes of their immunoglobulin gene restriction fragment patterns when analyzed longitudinally over time. These changes should not be confused with second or "biclinal" lymphomas and may serve as a marker for the acquisition of secondary growth promoting mutations.

Publications:

Stetler-Stevenson MA, Raffeld M, Cohen P, Cossman J. Detection of occult follicular lymphoma by specific DNA amplification. Blood 1988;72:1822-5.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09144-05 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Identification of Proteins Binding to c-myc Regulatory Sequences

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

PI:	D.L. Levens	Chief, Gene Regulation Section	LP NCI
OTHER:	M. Takimoto	Visiting Fellow	LP NCI
	M. Avigan	Medical Staff Fellow	LP NCI
	S. Pittaluga	Visiting Associate	LP NCI

## COOPERATING UNITS (if any)

Dr. M. Zajac-Kaye, Medicine Branch, DCBD, NCI

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Gene Regulation Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.25

## PROFESSIONAL:

2.75

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

B

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

Application of an exonuclease assay developed in this laboratory to identify and map the sites of tight protein-DNA interactions on large pieces of DNA, to the c-myc gene has revealed multiple cis- and trans-elements both upstream and downstream of the c-myc promoter P1. We have focused on 3 regions, each of which interacts with one or more sequence-specific binding proteins.

300 bp upstream of P1, fos/jun (AP1) and octamer binding proteins bind to a negative element. Approximately 1 kb downstream, a 140 kD phosphoprotein binds to a negative element.

1.5 kb upstream of P1, a cell-type and differentiation specific factor containing peptides of 22-30 kD, binds to a positive element.

A negative element has previously been localized to a 57-base pair segment approximately 300 base pairs upstream of the human c-myc promoter P1. Within this element, a 26-base pair region was protected in vitro from DNase I digestion with a HeLa cell nuclear factor(s). Two specific DNA-protein complexes were identified in gel retardation assays using HeLa cell nuclear extracts and an oligonucleotide probe spanning the footprinted region. Exonuclease and chemical footprint analyses suggested that the binding sites for both complexes are almost entirely overlapping. One of the complexes was eliminated by oligonucleotide competitors possessing known AP-1 binding sites. This same complex reacted strongly with anti-fos immunoglobulin suggesting a role for c-fos in governing c-myc expression. Precipitation of fos protein bound to c-myc DNA that was immobilized on beads confirmed the involvement of c-fos in a specific complex with the c-myc upstream sequence. In contrast, the other complex seen by the c-myc probe could not be competitively inhibited by AP-1 binding sites and was not affected by anti-fos antibody. Instead, this complex was efficiently eliminated by unlabeled oligonucleotides containing the octamer DNA motif found in immunoglobulin gene promoters. Purified octamer-binding proteins formed stable complexes with the 26-base pair c-myc sequences. These results demonstrate that degeneracy in the consensus recognition sequences of these distinct factors allows each of them to bind the c-myc negative element. The interaction of known transcriptional activators with a negative element suggests that the same factors can mediate both transcriptional activation and repression.

A 138 kD nuclear protein was identified from HeLa cells as the factor which binds to a previously described 20 bp cis element located in the intron I of the c-myc gene. This myc intron factor (MIF) binds to the wild type c-myc sequence but does not bind to c-myc from Burkitt's lymphoma which contain point mutations in this binding region. We have demonstrated that the 138 kD MIF is a phosphoprotein and that the treatment of the purified MIF with potato acid phosphatase abolished binding to its 20 bp c-myc recognition sequence; binding activity was protected by inclusion of phosphatase inhibitors. These results suggest that phosphorylation is required for the specific DNA:MIF interaction in vitro and that the phosphorylation state of MIF may be an important factor in controlling c-myc expression in vivo.

Using a sensitive exonuclease assay, we have identified sites of factor binding from whole cell extracts to genomic probes encompassing 2300 bases of c-myc sequence upstream P1. Protein extracts were prepared from undifferentiated as well as DMSO induced HL60 and U-937 cells in order to detect alterations in factor binding as c-myc transcriptional initiation is shut off. At one locus approximately 1600 bases upstream of P1, there is a DNA binding complex which is only present in undifferentiated cells and which dramatically decreases, correlating with commitment to differentiation. The factor complex is present in a variety of tumor lines, including HeLa cells, but not in some other lines including Hep G2, suggesting that the transactivating effect of FUSE is cell-type specific. We have constructed a CAT plasmid containing 3.2 kb of c-myc sequence including the far upstream element (FUSE) and the c-myc promoters fused to CAT. When transfected into U-937, HeLa, and Hep G2 cells, the plasmid expressed significant amounts of CAT. When a strategic 4 base deletion in the binding site of FUSE was made, there was a significant reduction of CAT expres-

sion in U-937 transfectants and HeLa cells but not Hep G2 transfectants, demonstrating that the element has a potentiating effect on expression when bound by the factor complex. Using oligonucleotide affinity chromatography, we have partially purified factors which bind to the FUSE. UV cross-linking studies demonstrate that several polypeptides from 22-30 kD bind to the FUSE in a cell line and differentiation specific manner.

#### Major Findings:

1. fos/jun (AP1) and octamer binding proteins interact with a negative element -300 relative to c-myc promoter Pl.
2. A 138 kd protein binds in a phosphorylation dependent manner to a cis-element frequently mutated in Burkitt lymphoma.
3. A positive cis-element at -1500 relative to Pl binds cell type and differentiation specific polypeptides of 22-30 kD.

#### Publications:

Takimoto M, Quinn JP, Farina AR, Staudt LM, Levens DL. fos/jun and Octamer-binding protein interact with a negative element of the human c-myc gene. J Biol Chem (in press)

Hay N, Takimoto M, Bishop JM. A fos protein is present in a complex that binds a negative regulator of myc. Genes & Development 1989;3:293-3.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09168-02 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Analysis of a Multiprotein Complex Interacting with the Gibbon Ape Leukemia

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

PI:	D.L. Levens	Chief, Gene Regulation Section	LP NCI
OTHER:	J. Quinn	Visiting Associate	LP NCI
	A. Farina	Guest Researcher	LP NCI
	K. Gardner	Biotechnology Fellow	LP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Gene Regulation Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

3.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not in itself possess full specificity. The second component does not in itself bind DNA, but upon forming a complex with the first component confers greatly enhancer power to discriminate between different sequences. These proteins are distinct and separable from fos/jun (AP1). However, a minor complex is present in MLA 144 which contains a fos-related antigen (FRA). The two components of the major complex can be independently activated in a cell-line specific manner.

At least two subunits contributed to the formation in vitro of a specific complex binding to the AP1 consensus sequence (TGAGTCA) in the gibbon ape leukemia virus (GALV) enhancer in MLA 144 cells. This complex can be dissociated on a monomeric GALV oligonucleotide affinity column. One protein, termed the "core protein", was retained on the oligonucleotide affinity column. The second protein flowed through the oligonucleotide affinity column and alone did not bind to DNA; however, with the core protein, the two bound strongly and very specifically to the GALV sequence. MLA 144 cells contained only trace amounts of c-fos and c-jun by Western blot analysis, suggesting that the proteins specifically binding to the GALV AP1 site were distinct from c-fos and c-jun. In addition to the major complex that recognized the GALV element, MLA 144 cells also contained a minor complex that is chromatographically different and antigenically related to c-fos. The factor in the flow-through complemented a human T-cell nuclear extract (Jurkat cell line), which alone had no assayable complex that specifically bound to the GALV enhancer; this complementation gave rise to a specific complex similar to that seen in MLA 144 cells. Together these results suggest that the GALV enhancer can interact with multi-component protein complexes in a cell line-specific manner. Purification of the separated components is virtually complete and the work in progress is concentrating on the generation of antibodies and clones to study the interaction of these proteins and to elucidate their regulation.

#### Major Findings:

1. A core complex composed of a medium-sized protein (approximately 35 kD) interacts with a second factor to produce a specific GALV/AP1 site binding complex. This complex is distinct from fos/jun.
2. A minor complex contains fos-related antigens.
3. The components of the major complex can be regulated in a cell line-specific manner.

#### Publications:

Quinn JP, Takimoto M, Iadarola M, Holbrook N, Levens DL. Distinct factors bind the AP-1 consensus sites in the gibbon ape leukemia virus and SV40 enhancers. *J Virol* 1989;63:1737-2.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09169-02 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Development of Leukocyte Cell-Substrate Adhesive and Migratory Ability

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

PI: G.E. Davis

Medical Staff Fellow

LP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## 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.)

Leukocytes have the ability to adhere and migrate in vitro and in vivo in response to various stimuli such as inflammatory mediators. In an attempt to study this process in molecular detail, I have chosen to work with HL-60 promyelocytic leukemia cells which are deficient in cell-substrate adhesive ability in the undifferentiated state. Previous work and my preliminary data indicate that they can be induced to become adhesive and migratory leukocytes when they are treated with phorbol esters alone or with combinations of other chemical agents and phorbol esters. Both the cell-substrate adhesion and migration observed with these treatments can be completely inhibited by a monoclonal antibody derived against the common beta subunit of the leukocyte integrin receptors (LFA-1, MAC-1, 150,95). Current experiments are focusing on the identification of the ligands involved in the adhesive events and on how phorbol esters are dramatically stimulating adhesive and migratory response. Leukocyte proteolytic enzymes are felt to play a role during the invasion of the endothelial basement membrane in vivo which occurs during inflammatory responses. A 94 kDa gelatinase from phorbol ester treated HL-60 cells has been isolated and characterized. The purified enzyme is latent and can be activated by a variety of agents including organomercurials and by denaturants such as acid, urea and SDS. During activation, an autocatalytic cleavage of peptide bonds occurs resulting in several lower molecular weight species. Amino acid sequences from the N-terminus and cyanogen bromide and tryptic peptide fragments have revealed unique sequences and a highly homologous sequence to other collagenase family enzymes. This data shows that this leukocyte gelatinase is a new member of collagenase family of metalloproteases.

Major Findings:

I have found that HL-60 cells can be used as a model for the development of leukocyte cell-substrate adhesive and migratory ability. They can be induced to adhere to tissue culture plastic substrata if treated with TPA alone (after 10-12 hours) or if first treated with DMSO or vitamin D3 for 48-72 hours followed by exposure to phorbol esters (TPA) for 30-60 minutes. In addition, these predifferentiated cells can be induced to strongly migrate in a Boyden-chamber apparatus if exposed to appropriate levels of TPA. Further support for the probable role of protein kinase C in the rapid effect of phorbol esters in cell adhesion is the finding that a distinct protein kinase C stimulator, DiC8, also induces the effect. In addition, the protein kinase C inhibitor, staurosporine, completely blocks the migratory response of predifferentiated cells in response to TPA. The goal of these studies is to understand the development of these cellular behaviors in molecular detail. The likely adhesive receptors involved in the phenomenon are those of the leukocyte integrin receptor family (LFA-1, MAC-1, 150,95) since a monoclonal directed to the common beta subunit of these receptors completely blocks the adhesive and migratory response. Recent experiments have been attempting to identify potential ligands for these receptors which are derived from the differentiating HL-60 cells. Once ligands are identified, experiments will address whether TPA effects the expression or function of the receptors and/or ligands.

During TPA treatment, HL-60 cells develop cell-substrate adhesive ability and also release a 94 kDa gelatin-degrading metalloprotease (gelatinase). The release of the gelatinase closely parallels the timing and extent of cell-substrate adhesion. It does not appear to be required for cell-substrate adhesion (blocking antibodies do not interfere with adhesion) and it is released at the same time whether or not the cells adhere to the substrate. The latter experiment was done using the blocking monoclonal antibody against the leukocyte integrin receptors mentioned above. By a number of criteria, the gelatinase is distinct from other collagenolytic metalloproteases and experiments were undertaken to obtain amino acid sequence data for the molecular cloning of the enzyme. In collaboration with Dr. Brian Martin (NIMH), we have obtained sequence data from the N-terminus and several internal sites. This data shows unique sequence as well as a highly homologous sequence with all other collagenase enzymes. Our data shows that this gelatinase is a new member of the collagenase family of metalloproteases. Future work will focus on the functional role of the gelatinase in leukocyte extravasation and in its genetic regulation during leukocyte development.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09170-02 LP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Identification of Genes Differentially Expressed in Developing Embryonic Limb Buds

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

PI: S. Mackem

Senior Staff Fellow

LP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

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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 elucidation and characterization of genes necessary for establishing pattern formation during morphogenesis and the study of their regulation are problems which are central to many aspects of vertebrate biology. It may be expected that processes including responses to trophic stimuli, cell-cell interactions, migration, differential cell multiplication, programmed cell death, etc. are all involved. Many of these same processes are recapitulated in a pathologic manner during oncogenesis. Chick limb development is an attractive system for studying the molecular basis for pattern formation during morphogenesis. Critical events at the level of tissue/cellular interactions involved in pattern formation have been well characterized in this organism, the events regulating pattern formation appear to be overall very similar to those in mammalian systems, and it is possible to isolate limb bud tissue from defined stages of development in large numbers. In addition, this system is readily amenable to studies involving microsurgical manipulation, and the recent availability of defective retroviral vectors provides a means for studying the effects of expression of cloned genes of interest in specific, selected tissues within the developing embryo. It is the aim of this long-term project to isolate genes that regulate morphogenesis in the chick embryo limb bud.

Major Findings:

Dual approaches are being employed to isolate genes involved in morphogenesis of the chick embryo limb bud. cDNA cloning and subtractive hybridization techniques are being used to identify genes that are differentially expressed at different stages of limb bud development and/or show specificity of expression with regard to limb type. A second, more directed approach will entail the use of cloned genes which have been implicated in developmental regulatory processes (especially pattern formation) in other systems as probes to screen chick limb bud cDNA libraries for related sequences. Genes that are identified using these approaches will be initially analyzed for their specific spatial and temporal patterns of expression in the embryo by the use of in situ hybridization. These studies may provide insight into factors involved in initiating or maintaining morphogenetic gradients, and clues as to possible hierarchies of interaction/interplay between different factors involved in regulatory circuits during pattern formation. The introduction of cloned genes of interest into defective avian retroviral vectors will provide a means for studying the effects of transient expression in specific tissues by localized injections into developing embryos, as well as potentially allowing for the development of transgenic animals. The isolation and characterization of corresponding genomic clones will also eventually provide the tools necessary to study the transcriptional and post-transcriptional regulation of genes determining pattern formation and of the "down stream" structural genes which they regulate to bring about the developmental program.

For the purpose of generating hybridization-subtraction probes, early stage (17-18) and late stage (21-22) limb bud mRNAs were extracted from about 2000 individually dissected chick embryos. These stages were chosen as likely to represent times at which signals regulating anterior-posterior and dorso-ventral patterning are just beginning to be expressed ("early"), and times at which morphogenetic gradients are well established ("late"). The isolation of differentially expressed mRNAs from these different stages should identify genes that are involved in establishing pattern formation, or at the very least, "marker" genes that are regulated by such morphogenetic signals. In addition, considering the profound differences in morphology between fore and hind limb buds in birds which is determined by the mesenchyme of the limb bud at a very early stage, wing and leg bud mRNA populations were extracted separately at the indicated stages, to allow identification of mRNAs differentially expressed in wing or leg limb buds.

Because of the relatively small amounts of mRNA that can be isolated even from a large number of limb buds (< 1 mm length each), and considering that regulatory signals may be expressed in only a small proportion of the total limb bud cell population at a given stage, techniques were sought to amplify limiting amounts of mRNA so that hybridization subtraction probes could be easily generated and to increase the likelihood of isolating low abundance sequences. The different mRNA populations were stably amplified via directional cDNA cloning into a  $\lambda$  bacteriophage vector ( $\lambda$  zap) in which the oriented cDNA insert is flanked by T3 and T7 bacteriophage promoters. The amplified cDNA population can then be transcribed to specifically yield large amounts of either sense or antisense RNA for use in the generation of hybridization subtraction probes. In addition, a modified polymerase chain reaction (pcr)

procedure using primers that flank the phage cDNA inserts is being developed, which would allow the generation of amplified single-stranded cDNA pools for use in subtractive hybridizations. This latter technique could be useful for selectively amplifying the single-stranded (differentially expressed) cDNA isolated after multiple rounds of subtractive hybridization to increase the yield of low abundance sequences.

To date, four different cDNA libraries have been constructed in  $\lambda$  zap using mRNA isolated from early (st 17-18) and late (st 21-22) stage wing and leg buds. Each library contains approximately  $10^7$  independent clones, ranging in size from 300 to 2600 bp. Modified procedures (as described above) are currently being developed and optimized for enhancing the isolation of differentially expressed, low abundance sequences from these libraries by subtractive hybridization techniques. Concomitantly, the libraries are also being screened for expression of genes which have been implicated in developmental regulation and pattern formation in other systems. Particularly, several approaches to identifying members of the homeodomain and pou-homeodomain gene families are being employed, including the use of homologous DNA probes in low stringency hybridizations, anti-peptide antibodies recognizing the highly conserved protein domains for immunoscreening, and degenerate oligonucleotide primers based on amino acid sequence for selective pcr amplification of gene fragments from pooled library cDNA. Such approaches will also be applied to screening for the presence of other genes that, although not as intensively studied to date, appear to play a role in developmental regulation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09171-06 LP

formerly Z01 CB 09274-05 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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	Expert	LP NCI
OTHER:	S. Irving	Guest Researcher	LP NCI
	K. Smith	Breast Cancer Fellow	LP NCI
	P. Davis	Breast Cancer Fellow	LP NCI

## COOPERATING UNITS (if any)

Ulrich Siebenlist, Laboratory of Immune Regulation, NIAID, NIH

## LAB/BRANCH

Laboratory of Pathology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

4.0

## OTHER:

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 investigating the consequences of mitogen mediated signals to T cells by isolating and characterizing genes that constitute the immediate early transcriptional response to these events. We have isolated over 60 novel cDNA clones that represent mRNA species induced by PHA and PMA in human peripheral blood T cells. Primary sequence analyses on five clones have been completed and have revealed two functional classes of proteins encoded by these genes: lymphokines and DNA binding proteins/transcription factors. Lymphokines are likely important in regulating the proliferation and or differentiation of lymphoid and/or nonlymphoid cell types during an immune response. Transcription factors potentially affect "downstream" gene expression, and thus, can act as pleiotropic regulators of T cell activation. pAT 744 and pAT 464 are hematopoietic cell specific induced genes. They encode distantly-related members of a family of small secreted proteins, many of which appear to play roles in the inflammatory response and wound healing. pAT 469 and pAT 555 share nearly identical coding regions but represent alternatively-spliced 5' and 3' nontranslated regions. pAT 744 and 464/555 are linked on human chromosome 17. pAT 237 also is hematopoietic cell-specific and likely encodes a previously undescribed lymphokine with an 18.5 kd polypeptide backbone. Potential functional activities of the three putative lymphokines currently are being tested with recombinant proteins. pAT 225 and pAT 416 are responsive to signals in a variety of cell types. The pAT 225 encoded protein contains 3 adjacent zinc finger motifs and most likely is a DNA binding protein/transcription factor. pAT 416 encodes a protein which belongs to the steroid receptor/DNA binding class of proteins.



Major Findings:

The goal of this project is to broaden our understanding of the physiology and regulation of mitogen-induced activation. To this end we have isolated and are characterizing genes that are induced rapidly following mitogen stimulation of human peripheral blood (PB) T cells. We have employed the techniques of subtractive cDNA cloning coupled with using a labelled subtracted probe to select genes that are transcriptionally induced in PB T cells within four hours following the addition of PHA and PMA in the presence of cycloheximide. We have isolated in excess of 60 unique genes that are unrelated as determined by cross-hybridization analyses. Greater than 90 percent of the clones represent previously undescribed induced genes. The clones demonstrate heterogeneity with regard to kinetics of induction, ranging from 30 minutes to 4 hours, and levels of expression, encompassing less than 10 to greater than 100 induced copies per cell. Analyses of the large number of induced clones that have been isolated offer the opportunity to begin elucidating the complexity of mitogen-induced responses. Two important broad areas of interest are the physiological functions of induced genes and the spectrum of regulatory mechanisms controlling induced gene expression.

Primary Structure Determination

The determination of primary structure is an essential step in the analysis of an induced gene which can indicate a class of protein such as a membrane receptor, secreted protein, DNA-binding protein, protein kinase, or calcium-binding protein. We have completed or are determining the complete nucleotide structure of ten full-length cDNA clones encoding inducible mRNA's. Four of these genes (pAT 464, pAT 555, pAT 744, pAT 237) are not induced in fibroblasts but instead appear to be specific to hematopoietic tissue. Seven of these genes (pAT 464, pAT 744, pAT 237, pAT 120, pAT 255, pAT 154, and pAT 416) are expressed at high constitutive levels in adult T cell leukemias (ATL) and HTLV I infected human T cell clones. Five of these genes (pAT 464, pAT 744, pAT 154, pAT 237 and pAT 555) are transcriptionally inhibited by cyclosporin A. pAT 125 and pAT 127 are genes that encode abundant mRNA's, and thus were isolated with a high frequency from the subtracted library. As can be seen, many of the 10 genes selected for detailed analyses demonstrate more than one interesting property. Complete sequence has been obtained for pAT 225, pAT 744, pAT 555, pAT 464, pAT 416, and pAT 237. Sequencing is in progress for the remaining clones.

pAT 464, pAT 744, and pAT 555 are hematopoietic cell-specific induced genes. The complete translated amino acid structures of pAT 744, pAT 464, and pAT 555 indicate that these genes encode proteins of approximately 8 kdaltons which are related to varying degrees, including the important finding that each contains an apparent hydrophobic leader sequence but no obvious transmembrane domain, suggestive of secreted proteins. pAT 464 and pAT 555 represent independent isolates from a cDNA library probed with the original pAT 464 clone from the subtracted library. They are greater than 90% homologous in the major part of the coding regions of the genes with distinct 5' and 3' ends. Oligonucleotide probes specific for the 555 and 464 5' difference hybridize to adjacent first exons on a genomic clone, implying that the two cDNA's represent alternatively transcribed and/or spliced products. pAT 464/555 are approximately 55% homologous at the amino acid level with pAT 744. Anti-peptide antibodies specific

for pAT 464 or 744 detect secreted proteins in the supernatants of activated T cell cultures or COS cells transfected with expression vectors containing the 464 and 744 gene sequences. The proteins encoded by these three genes share some critical amino acid similarity with a newly emerging family of secreted factors, some of which have been shown to display functions associated with an inflammatory response and/or have mitogenic activities.

pAT 237 is a hematopoietic cell-specific induced gene. The predicted protein encoded by pAT 237 is 18.5 kdaltons, cysteine-rich, contains an apparent N-terminal hydrophobic leader sequence, no transmembrane domain, and a potential N-linked glycosylation site. Thus, it is highly likely that pAT 237 encodes a lymphokine. In addition, the cDNA features a 400 b.p. 5' non-translated region and five out-of-frame ATG codons. Such a structure is uniquely characteristic of lymphokine and oncogene messages. pAT 237 demonstrates no homology to known lymphokines although the cysteine-rich nature of the protein is reminiscent of the T cell growth factor, p40 (Van Snick et al. 1989, JEM 169:363). The biochemical properties of this protein are currently being assayed using two anti-peptide antibodies derived from amino- and carboxy-terminal regions of the protein.

pAT 225 was the most frequently isolated gene in the subtracted library, and it is induced in both growth-activated fibroblasts and T cells. pAT 225 encodes a predicted protein of 56 kdaltons, and importantly, this protein contains a region with 3 apparent zinc finger structures. pAT 225 demonstrates approximately 85% nucleic acid homology to a gene isolated from both serum-treated murine 3T3 cells (EGR-1, Krox 24) and nerve growth factor-treated rat PC-12 cells (NGF-1a), and thus is likely the human homologue of the previously-described rodent genes. The association of zinc finger structures in other genes with DNA binding activity and transcriptional activation of specific target genes strongly suggests that pAT 225 encodes a transcription factor.

pAT 416 is induced in both growth factor activated fibroblasts and in mitogen-treated T cells. pAT 416 appears to encode a DNA binding protein similar to steroid receptors.

#### Gene Regulation Studies

We have chosen pAT 464 and 744 to initiate gene regulation studies. We anticipate that these genes will encode functionally interesting proteins. We have isolated multiple genomic clones for pAT 464 and 744. Mapping of these clones has revealed that the two genomic loci are linked; i.e. separated in the genome by approximately 8 kilobases. The location of exons within the pAT 464 and 744 genomic loci has been established by the use of oligonucleotide probes and subsequent sequencing. Approximately 1.2 kilobases of sequence has been determined 5' to the first exon of pAT 744. This sequence features a canonical TATA and CAT box in addition to a consensus cAMP responsive element and multiple elements commonly found upstream in lymphokine genes (PuPuPuPuTTPyCAPy). The pAT 744 upstream sequence has been connected to a CAT reporter gene. Such a hybrid gene shows appropriate inducibility following treatment of transfected Jurkat cells with activating agents.

Publications:

Zipfel PF, Irving SG, Kelly K, Siebenlist U. Complexity of the primary transcriptional response to mitogenic activation of human blood T cells. *Mol Cell Biol* 1989;9:1041-8.

Irving SG, June CH, Zipfel PF, Siebenlist U, Kelly K. Mitogen induced genes are subject to multiple pathways of regulation in the initial stages of T cell activation. *Mol Cell Biol* 1989;9:1034-0.

Gunter KC, Irving SG, Zipfel PF, Siebenlist U, Kelly K. Cyclosporin A-mediated inhibition of mitogen-induced gene transcription is specific for the mitogenic stimulus and cell type. *J Immunol* (in press, May 1989)

Zipfel PF, Balke J, Irving SG, Kelly K, Siebenlist U. Characterization and regulation of two T-cell induced genes which are members of a newly emerging family of inflammatory response genes. *J Immunol* 1989;142:1582-0.

SUMMARY STATEMENT  
ANNUAL REPORT  
DERMATOLOGY BRANCH  
DCBD, NCI

October 1, 1988 through September 30, 1989

The Dermatology Branch conducts both clinical and basic research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and the host's response to these diseases. The basic research involves biochemical as well as biological studies of skin and is subdivided into six separate, though frequently interacting, areas. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 1500 patients are seen in consultation each year). The main research achievements of the Dermatology Branch for the past year are as follows:

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases:

We have continued our studies of the immunological functions of cells of the epidermis. We have found that when murine 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. Utilizing these cells in primary immune responses in vitro, we found that the T cells responded preferentially to the hapten to which they were "primed." We have produced T cell lines from these in vitro sensitized cells and have found that during the first few stimulations the primed T cells produce mainly IL-2, however after multiple stimulations they produce mainly IL-4 and not IL-2. Utilizing this in vitro sensitization system, we have recently attempted to generate primary in vitro responses to tumor-associated antigens. We have utilized the Friend Leukemia Virus associated gp-70 protein but have been unable to detect any antigen processing or presentation by cultured or freshly prepared epidermal Langerhans cells.

We have also initiated studies in human beings in which we will attempt to generate primary T cell responses in vitro. We have demonstrated that cultured Langerhans cells exhibit increased class II MHC molecules and are potent alloantigen presenting cells. We will assess their ability to present protein antigens and then attempt to generate primary in vitro responses to viral-or tumor-associated antigens.

Studies assessing the effects of cyclosporine A (CSA) on antigen presenting and accessory cell functions of Langerhans cells have demonstrated that CSA interacts with Langerhans cells after a 2 hour incubation and renders them (Langerhans cells) relatively unable to perform many of the T cell stimulatory functions. In addition, CSA has a profound effect on the proliferation of epidermal cells. We have also studied the effects of dexamethasone on epidermal Langerhans cells. From these in vitro studies, it appears that dexamethasone is cytolytic to a subpopulation of Langerhans cells. In studies



comparing the effects of dexamethasone and cyclosporine on the activation of T cells, we have demonstrated a differential effect of the two. That is, using various activation signals, it is clear that phorbol and ionomycin activation of T cells cannot be inhibited by dexamethasone, whereas it is completely blocked by cyclosporine.

#### Molecular Basis of Autoimmune Skin Diseases:

This laboratory studies autoantibody-mediated skin diseases in order to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis and epidermal basement membrane zone. Specifically, antibodies in these diseases define molecules in the normal epidermis. We have characterized the antigens defined by antibodies in the sera of patients with three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV) and pemphigus foliaceus (PF).

We have determined, with immunochemical methods, that the BP antigen is a 230 kD protein with a pI of 8. The sera of almost all patients with BP bind to this polypeptide, and, therefore, immunoprecipitation can be used as a diagnostic technique. With antibody screening of a  $\lambda$ gt11 expression cDNA library derived from cultured human keratinocytes, we have isolated a cDNA clone encoding the C-terminal end of this antigen. We are using this cDNA as a probe to find the full length cDNA for BP antigen. The encoded peptide is not homologous to any known protein.

We have also characterized, by immunochemical methods, the PV and PF antigen complexes extracted from normal human epidermis, and shown that they contain the adhering junction molecules desmoglein (PF antigen) and plakoglobin (both antigens). Thus, in diseases of defective cell adhesion, autoantibodies are directed against adhering junction components.

#### Molecular and cell biology of immunoreactive molecules in the Skin:

We have recently established a laboratory whose primary purpose is to elucidate the molecular mechanisms regulating the expression of immunoreactive molecules in the skin. ICAM-1, or intercellular adhesion molecule-1, is a cell surface protein that is the major ligand for LFA-1, or lymphocyte function associated antigen-1, an anchoring receptor on all T cells whose interaction with ligand is critical for proper T-cell function. Cutaneous T-cell inflammatory and neoplastic diseases may depend upon the regulation of ICAM-1 expression by various cells in the skin. We have established that human keratinocytes (HK) in culture do not normally express ICAM-1, and that exposure of HK to human interferon-gamma (IFN) or tumor necrosis factor-alpha (TNF), two biologic response modifiers (BRM), results in the rapid induction of ICAM-1 mRNA expression, protein biosynthesis, and surface expression.

We have also demonstrated that human dermal microvascular endothelial cells (HDMEC) constitutively express ICAM-1, that this expression can be rapidly upregulated both at the level of mRNA and in surface expression by exposure to human interleukin-1 (IL-1). We have also found that specific T-cell binding to HDMEC in vitro is dependent upon ICAM-1 expression by HDMEC and can be upregulated as well by factors which increase ICAM-1 expression.

We have established that ICAM-1 expression in HK, and HDMEC is regulated at the level of gene transcription and we have isolated a 19 kb genomic clone containing portions, if not all, of the ICAM-1 gene. Mapping and sequencing of the 5' flanking regulatory region of this genomic clone of ICAM-1 has been partially completed.

#### 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 as well as in the prevention of cancer.

Seven patients with xeroderma pigmentosum were entered into a cancer chemoprevention study using isotretinoin: results indicate significant partial inhibition of new tumor formation during therapy and a marked increase in new tumor formation after therapy. Long-term therapy with low-dose isotretinoin is now being used for cancer chemoprevention in these patients. The need for continuous maintenance therapy for chemoprevention of skin cancer with isotretinoin may vary with the underlying etiology (genetic or environmental) of the patient's tumors.

Retinoid hyperostosis, a variant form of diffuse idiopathic skeletal hyperostosis, was observed in patients with multiple basal cell carcinomas treated with high doses (1.5 mg/kg/day or higher) of isotretinoin for a minimum of 2 years. Severity of this disorder, defined by anterior spinal ligament calcification and osteophyte formation at 2 or more vertebral levels without disc space narrowing, appeared to be dose-dependent. Isotretinoin induced calcification of both spinal ligaments and extraspinal ligaments and tendons. Sixty percent of acne patients with moderate doses of isotretinoin for 9 months also developed vertebral osteophytes. Extraspinal tendon and ligament calcification also occurred commonly after chronic therapy for psoriasis and disorders of keratinization with etretinate, an aromatic retinoid. The usual sites of involvement were the ankles, pelvis and knees.

#### Studies of DNA Repair in Normal Human Cells from Patients with Xeroderma Pigmentosum and Neurodegenerative Disorders:

We are continuing our studies of the role of DNA repair processes in cancer, aging and neurodegenerative disorders. In collaboration with Dr. Kenneth H. Kraemer, we are continuing to transfect human lymphoblastoid cell lines with a plasmid (pRSVcat) which contains the bacterial cat gene coding for chloramphenicol acetyltransferase. The plasmid is previously irradiated with different doses of 254-nm UV. Then, in order to evaluate the repair of dimer and nondimer photoproducts, aliquots containing the irradiated plasmid are treated with photoreactivating enzyme (*E. coli* photolyase) and light, a treatment which monomerizes the UV-induced cyclobutane-type of pyrimidine dimer and leaves only nondimer photoproducts. We now have evidence that at least some Cockayne syndrome fibroblast strains, like XP fibroblast strains, are unable to repair not only pyrimidine dimers but also nondimer photoproducts induced in the plasmid by UV.

In collaboration with Dr. Vilhem A. Bohr and Dr. Michele K. Evans, we are studying the repair of ultraviolet radiation (UV-induced cyclobutane pyrimidine dimers in an active housekeeping gene (the dihydrofolate reductase [DHFR] gene) as opposed to the conventional study of repair in the genome overall. In this procedure, isolated DNA from fibroblasts is restricted, separated from replicated DNA, nicked at dimers with T-4 endonuclease V, electrophoresed in alkaline agarose, and subjected to Southern hybridization. We have studied normal and XP group A, C, and F fibroblast strains. We have found all the XP strains to have defective removal of dimers from the DHFR gene. The cells' relative capacity to repair the gene is the same as their relative capacity to perform UV-induced unscheduled DNA synthesis in the genome overall and does not correlate with the cells' relative ability to survive the lethal effects of UV. These studies show that removal of dimers from an active gene is not required for high survival, since the relatively UV-resistant group F strain showed virtually no dimer removal. Our results indicate that some lesions(s) other than dimers are likely to be important in UV lethality. We plan to adapt the active-gene-repair assay to monitor the repair of nondimer lesions induced by UV, for example, the 6-4 photoproduct. We plan also to study active gene repair in Cockayne syndrome cells in an attempt to confirm reports of others that in Cockayne syndrome there is defective DNA repair in active genes but not in the genome overall.

#### Chemistry, Structure, and Biosynthesis of Mammalian Epidermal Keratin Filaments:

We are continuing our studies on the biosynthesis, structure and functions of epidermal cytoskeletal proteins, the principal products of human and mouse epidermis. We are using NMR techniques to study the nature of the molecular interaction of keratin filaments and filaggrin, which is an important filament-associated protein in the epidermis. Keratin filaments isotopically labeled with glycine (for end domains) and glutamic acid or lysine (for rod domains) are complexed with filaggrin. Changes in the molecular motions of the filaments will provide information on their mode of interaction with filaggrin. Preliminary data suggests the molecular motions of the glycine regions are unchanged, implying indirectly that filaggrin interacts with the rod domains. Experiments in progress with lysine -or glutamic acid- labeled filaments should provide more direct evidence for this mechanism.

Many cDNA clones to human filaggrin have now been isolated and sequenced. The human filaggrin gene encodes a polyprotein containing a large number of filaggrin repeats arranged in tandem. Sequencing has shown that these repeats are highly polymorphic but conserved in size. Also, the gene from different individuals contain differing numbers of repeats. In collaboration with Dr. Lessin, we have localized the human gene to chromosome 1q21 region. Attempts are now underway to isolate the human gene in order to define regulatory sequence regions by use of CAT assays and eventually production of transgenic mice bearing the human gene.

We have isolated, cloned and sequenced the gene for the major human cornified cell envelop proteins, named loricrin. Fragments of this gene are now being subcloned into CAT vectors to define regulatory regions. In collaboration with Dr. Dennis Roop, the clones bearing the complete gene will be used for

the production of transgenic mice. In collaboration with Dr. Ron Chung, we have begun to isolate and characterize cDNA clones for epidermal transglutaminase, the enzyme which cross-links the loricrin to the cell envelop. In addition, we have synthesized peptides corresponding to loricrin sequences for in vitro transglutaminase assays, and <sup>3</sup>H-lysine-labeled cross-linked peptides of loricrin from labeled foreskins will be characterized to define the structural organizational of loricrin in the cell envelop.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03657-15 D

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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: A. Gaspari, Medical Staff Fellow, Dermatology Branch, DCBD, NCI  
 T. Furue, Visiting Fellow, Dermatology Branch, DCBD, NCI  
 S. Aiba, Visiting Fellow, Dermatology Branch, DCBD, NCI

## COOPERATING UNITS (if any)

Dermatology Branch, USUHS, Bethesda  
 Immunology Branch, DCBD, NCI

## LAB/BRANCH

Dermatology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5

## PROFESSIONAL:

4

## OTHER:

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 major area of study of this laboratory is the role of the epidermis as an immunological organ. We have found that epidermal Langerhans cells are derived from precursor cells in the bone marrow and play an essential role in many of the immunological reactions affecting the skin. We have also identified an epidermal Interleukin 1-like cytokine which may serve as a second signal in the generation of T cell responses as well as a proinflammatory agent affecting other cells - especially neutrophils. We have demonstrated that when murine 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 haptens, they can generate primary immune responses. The sensitized T cells thus generated respond preferentially to the same hapten in vitro. We have, therefore, attempted to utilize this system for the generation of primary in vitro responses to tumor-associated antigens such as the Friend Leukemia Virus associated gp-70. We are also currently assessing the feasibility of performing these studies in cells derived from human beings. Preliminary studies demonstrate that cultured human Langerhans cells have enhanced antigen presenting capacity. The other major focus of this laboratory has been the study of the function of class II MHC bearing keratinocytes which appear in humans and mice during cell-mediated reactions in the skin. We have demonstrated that these cells can 1) present peptide fragments to T cell hybridomas, 2) serve as targets for class II specific cytotoxic T lymphocytes, and 3) induce secondary alloreactive T cell responses, although the first and third are performed only poorly. Our recent studies demonstrate that these class II bearing keratinocytes induce specific immunological unresponsiveness in cloned T cells and in vivo in contact sensitized mice.

Project DescriptionMajor Findings:

We have found that when murine 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. Utilizing these cells in primary immune responses in vitro, we found that the T cells responded preferentially to the hapten to which they were "primed." We have produced T cell lines from these in vitro sensitized cells and virtually all of them produce IL-4 as opposed to IL-2. We have found that during the first few stimulations, the primed T cells produce mainly IL-2, however after multiple stimulations they produce mainly IL-4 and not IL-2. Utilizing this in vitro sensitization system, we have recently attempted to generate primary in vitro responses to tumor-associated antigens. We have utilized the Friend Leukemia Virus associated gp-70 protein but have been unable to detect any antigen processing or presentation by cultured or freshly prepared epidermal Langerhans cells.

We have also initiated studies in human beings in which we will attempt to generate primary T cell responses in vitro. We have demonstrated that cultured human Langerhans cells exhibit increased class II MHC molecules and are potent alloantigen presenting cells. We will assess their ability to present protein antigens and then attempt to generate primary in vitro responses to viral-or tumor-associated antigens.

We have continued our studies of the murine thy 1<sup>+</sup> dendritic epidermal cells which are bone marrow derived. Using freshly isolated cells in C3H mice we found that they expressed the protein products of the  $\gamma\delta$ -like T cell receptor heterodimers and the associated T3 components. These findings conclusively characterize these cells of being of T cell lineage. We are currently assessing the role of the epidermis in the expression of the T cell receptor-T3 complex.

Other major interests and studies include 1) those characterizing the functions of class II-MHC bearing keratinocytes, and 2) the assessment of the effect of cyclosporine A on the antigen-presenting and accessory cell functions of Langerhans cells and on the activation of T cells. With regard to the former, we have developed techniques which can deplete Langerhans cells from class II bearing keratinocytes so that the function of these keratinocytes, which are induced by injecting interferon gamma, can be assessed. We found that they function 1) as targets for class II specific cytotoxic T cells, 2) as weak stimulators in secondary allogeneic T cell proliferative responses, and 3) poorly in the presentation of small peptide antigens to T cell hybridomas. We have also demonstrated that these class II bearing keratinocytes when hapten-coupled, induce specific immunological unresponsiveness in hapten-specific T cell clones. Our most recent studies indicate that class II bearing keratinocytes can induce tolerance to various haptens in vivo.

Studies assessing the effects of cyclosporine A (CSA) on antigen presenting and accessory cell functions of Langerhans cells have demonstrated that CSA interacts with Langerhans cells after a 2 hour incubation and renders them (Langerhans cells) relatively unable to perform many of the T cell stimulatory functions. In addition, CSA has a profound effect on the proliferation of epidermal cells. We have also studied the effects of dexamethasone on epidermal Langerhans cells. From these in vitro studies, it appears that dexamethasone is cytolytic to a subpopulation of Langerhans cells. In studies comparing the effects of dexamethasone and cyclosporine on the activation of T cells, we have demonstrated a differential effect of the two. That is, using various activation signals, it is clear that phorbol and ionomycin activation of T cells cannot be inhibited by dexamethasone, whereas it is completely blocked by cyclosporine.

Publications:

Sakamoto K, Sachs DH, Shimada S, Popitz-Bergez, FA, Pennington, LR, Pescovitz, MD, McDonough MA, MacVittie TJ, Katz SI, Gress RE. Bone marrow transplantation in miniature swine, Transplantation 1988;45:869-875.

Furie M, Katz SI. Molecules on activated human T cells, Am J Dermatopath 1988;10:349-355.

Hauser C, Katz SI. Activation and expansion of hapten- and protein-specific T helper cells from nonsensitized mice, Proc Nat Acad Sci 1988;85:5625-5628.

Gaspari AA, Jenkins MK, Katz SI. Class II major histocompatibility complex bearing keratinocytes induce antigen specific unresponsiveness in hapten-specific Th<sub>1</sub> clones, J Immunol 1988;141:2216-2220.

Aberer W, Katz SI. Effects of in vivo administration of anti-Ia antibodies on contact sensitivity, Arch Derm 1989;125:280-284.

Hauser C, Snapper CM, Ohara J, Paul WE, Katz, SI. T helper cells grown with hapten-modified cultured Langerhans' cells produce interleukin 4 and stimulate IgE synthesis, Eur J Immunol (in press).

Furie M, Katz SI. Direct effects of glucocorticosteroids on epidermal Langerhans cells, J Invest Derm 1989;89:342-347.

Kusuda M, Gaspari AA, Chan C -C, Gery I, Katz SI. Expression of Ia antigen by ocular tissues of mice treated with interferon gamma, Investigational Ophthalmology and Visual Science (in press).

Hall RP, Sanders ME, Duquesnoy RJ, Katz SI, Shaw SS. Alterations in HLA-DP and -DQ antigen frequency in patients with dermatitis herpetiformis, J Invest Derm (in press).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03667-05 D

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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., Medical Officer, Dermatology Branch, DCBD, NCI

OTHER: Neil Korman, M.D., Medical Staff Fellow, Dermatology Branch, DCBD, NCI  
Toshihiro Tanaka, M.D., Visiting 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:

4

## PROFESSIONAL:

3

## OTHER:

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 are characterizing the antigens defined by three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF).

We have determined, with immunochemical methods, that the BP antigen is a 230 kD protein with a pI of 8. Almost all patients with BP bind to this polypeptide, and, therefore, immunoprecipitation can be used as a diagnostic technique. With antibody screening of a  $\lambda$ gt11 expression cDNA library derived from cultured human keratinocytes, we have isolated a cDNA clone encoding the C-terminal end of this antigen. We are using this cDNA as a probe to find the full length cDNA for BP antigen.

We have also characterized, by immunochemical methods, the PV and PF antigen complexes extracted from normal human epidermis, and shown that they contain the adhering junction molecules desmoglein (PF antigen) and plakoglobin (both antigens). Thus, in diseases of defective cell adhesion, autoantibodies are directed against adhering junction components.



Project Description

Major Findings:

Bullous pemphigoid (BP) antigen is a 230 kD protein with pI of 8.

A cDNA encoding the C-terminal 1/2 of BP antigen has been isolated. By epitope selection, antibodies that bind the polypeptide encoded by this clone also bind the 230 kD antigen. The encoded peptide is not homologous to any other known basement membrane (or other) proteins.

Antibodies from pemphigus foliaceus (PF) patients bind a complex of polypeptides that includes desmoglein, a core desmosomal glycoprotein, and plakoglobin, a protein found in the dense plaques of both desmosomes and adherens junctions.

Antibodies from pemphigus vulgaris (PV) patients bind a complex of polypeptides that contains a 130 kD glycoprotein and plakoglobin.

The diagnosis of PF and PV can be made by immunoprecipitation of their respective antigens.

Publications:

Stanley JS: The enigma of fogo selvagen. J Am Acad Dermatol 1989;20:675-676.

Koulu L, Stanley JR: Clinical, histologic and immunopathologic comparison of pemphigus vulgaris and pemphigus foliaceus. Seminars in Dermatol 1988;7:82-90.

Parodi A, Stanley JR, Ciaccio M, Rebora A: Epidermal antigens in pemphigus vegetans. Br J Dermatol 1988;119:799-802.

Stanley JR, Tanaka TT, Mueller SM, Klaus-Kovtun V, Roop D: Isolation of cDNA for bullous pemphigoid antigen by use of patients' autoantibodies. J Clin Invest 1988;82:1864-1870.

Mueller S, Klaus-Kovtun V, Stanley JR: A 230-kD basic protein is the major bullous pemphigoid antigen. J Invest Dermatol 1989;92:33-38.

Matsuoka LY, Wortsman J, Stanley JR: Epidermal autoantibodies in erythema multiforme. J Am Acad Dermatol (in press).

Stanley JR: Pemphigus and pemphigoid as paradigms of organ-specific autoantibody-mediated diseases. J Clin Invest 1989;83:1443-1448.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03659-15 D

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Therapy of Skin Cancer, Disorders of Keratinization, and Cystic Acne

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

P.I.: G.L. Peck, Senior Investigator, Dermatology Branch, DCBD, NCI

OTHER: J.J. DiGiovanna, Expert Scientist, Dermatology Branch, DCBD, NCI  
 E. Toombs, Medical Staff Fellow, Dermatology Branch, DCBD, NCI  
 I. Tokar, Registered Nurse, Dermatology Branch, DCBD, NCI  
 K. Kraemer, Senior Investigator, Lab. Molecular Carcinogenesis

## COOPERATING UNITS (if any)

Cancer Prevention Studies Branch, DCPC, NCI, NIH, Bethesda, Maryland 20892

## LAB/BRANCH

Dermatology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.8

## PROFESSIONAL:

3.8

## OTHER:

## 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.)

Seven patients with xeroderma pigmentosum were entered into a cancer chemoprevention study using isotretinoin: results indicate significant partial inhibition of new tumor formation during therapy and a marked increase in new tumor formation after therapy. Long-term therapy with low-dose isotretinoin is now being used for cancer chemoprevention in these patients. The need for continuous maintenance therapy for chemoprevention of skin cancer with isotretinoin may vary with the underlying etiology (genetic or environmental) of the patient's tumors. Retinoid hyperostosis, characterized by anterior spinal ligament calcification and osteophyte formation of vertebrae and extraspinal tendon and ligament calcification, has been observed in patients treated with long-term, high-dose isotretinoin. Sixty percent of acne patients with moderate doses of isotretinoin for 9 months also developed vertebral osteophytes. Due to storage in fat, serum levels of etretinate can be measured for prolonged periods (> 2 years) after discontinuation of therapy.

Suicidal ideation is a potential problem in patients with chronic disfiguring dermatoses, such as Darier's disease. Seven of 11 patients with Darier's disease had a history of suicidal thoughts, plans or attempts. In a control group of 11 patients with lamellar ichthyosis and similar disorders, 3 patients had suicidal thoughts or plans.

One patient with multiple skin cancers developed an adverse drug reaction to fenretinide, a synthetic retinoid. The reaction included diarrhea, elevated liver function tests and a morbilliform skin eruption.

Project DescriptionMajor Findings:

Two patients with a history of multiple basal cell carcinomas were treated with oral isotretinoin for 7-8 years with inhibition of new tumor formation. After treatment was stopped in order to determine the need for chronic therapy, one patient with the nevoid basal cell carcinoma syndrome developed 29 tumors within 13 months. The second patient with arsenic-induced tumors developed one new tumor as of the 42-month visit. This indicates that the need for continuous maintenance therapy for chemoprevention of skin cancer with isotretinoin may vary with the underlying etiology (genetic or environmental) of the patients tumors. The efficacy of isotretinoin at high dose as a chemopreventive agent is being studied further in a series of 7 patients with xeroderma pigmentosum and nevoid basal cell carcinoma syndrome. Preliminary results indicate significant partial, but not complete, efficacy in inhibiting new tumor formation. Furthermore, impressive increases in the rate of new tumor formation have been observed after stopping therapy. Low-dose isotretinoin is now being used in these patients in order to minimize toxicity.

Retinoid hyperostosis, a variant form of diffuse idiopathic skeletal hyperostosis, was observed in patients with multiple basal cell carcinomas treated with high doses (1.5 mg/kg/day or higher) of isotretinoin for a minimum of 2 years. Severity of this disorder, defined by anterior spinal ligament calcification and osteophyte formation at 2 or more vertebral levels without disc space narrowing, appeared to be dose-dependent. Isotretinoin induced calcification of both spinal ligaments and extraspinal ligaments and tendons. Extraspinal tendon and ligament calcification also occurred commonly after chronic therapy for psoriasis and disorders of keratinization with etretinate, an aromatic retinoid. The usual sites of involvement were the ankles, pelvis and knees.

Detectable serum concentrations of etretinate were observed for more than two years following discontinuation of therapy. The median half-life of etretinate in the serum was calculated to be 12.5 weeks - overweight patients tended to have shown elimination, maintain higher serum concentrations and clear etretinate later.

Suicidal ideation is a potential problem in patients with chronic disfiguring dermatoses, such as Darier's disease. Seven of 11 patients with Darier's disease had a history of suicidal thoughts, plans or attempts. In a control group of 11 patients with lamellar ichthyosis and similar disorder, 3 patients had suicidal thoughts or plans.

One patient with nevus comedonicus covering most of the left side of his trunk had an inadequate response to isotretinoin but responded excellently to topical ammonium lactate lotion.

One patient with multiple skin cancers developed an adverse drug reaction to fenretinide, a synthetic retinoid. The reaction included diarrhea, elevated liver function tests and a morbilliform skin eruption.

Publications:

Sarnoff DS, Peck GL. Therapy of basal cell carcinoma. In: Magrath IT, ed. New Directions in Cancer Treatment. New York: Springer-Verlag, 1989;541-545.

Milton GP, DiGiovanna JJ, Peck GL. Treatment of nevus comedonicus with ammonium lactate lotion, J Am Acad Dermatol 1989;20:324-328.

Kraemer KH, DiGiovanna JJ, Moshell AN, Tarone RE, Peck GL. Prevention of skin cancer with oral 13-cis-retinoic acid in xeroderma pigmentosum, New Eng J Med 1988;318:1633-1637.

Peck GL, DiGiovanna JJ, Sarnoff DS, Gross EG, Butkus D, Olsen T, Yoder F. Treatment and prevention of basal cell carcinoma with isotretinoin, J Am Acad Dermatol 1988;19:176-185.

DiGiovanna JJ, Zech LA, Ruddel ME, Gantt G, Peck GL. Etretinate: persistent serum concentrations after long-term therapy, Arch Dermatol 1989;125:246-251.

Wolfish PS, Peck GL. Discussion of Problems 27-35: Retinoid Therapy. In: Problems in Dermatologic Diagnosis and Management. Lambert WC, ed. Evanston, American Academy of Dermatology, 1988;160-161.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03630-19 D

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (90 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, Expert Scientist, Dermatology Branch, DCBD, NCI  
 E. Toombs, Medical Staff Fellow, Dermatology Branch, DCBD, NCI  
 P.M. Steinert, Senior Investigator, Dermatology Branch, DCBD, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Dermatology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## 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 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 alltrans retinoic acid, but not systemic 13-cis-retinoic acid, increased gap junction density and decreased desmosome density in treated basal cell carcinomas. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

A specific cytosol retinol binding protein (CRBP) has been identified in normal human skin, newborn mouse skin and human skin from patients with Darier's disease, psoriasis and basal cell carcinomas. A specific cytosol retinoic acid binding protein (CRABP) has also been identified in newborn mouse and normal human adult skin and newborn foreskin. The qualitative and quantitative distribution between the epidermis and dermis of both CRBP and CRABP has been determined in adult human lower limb skin.

Using molecular hybridization probes specific for transcripts of individual keratin genes, keratin gene expression in skin cancer and cutaneous disorders of keratinization indicate the following. In contrast to normal epidermis where the expression of proliferation-associated keratin genes is suppressed after cells migrate from the basement membrane, hyperproliferative disorders of the epidermis exhibit inappropriate expression of proliferation-associated keratin genes in the more superficial layers of the epidermis. In addition, skin cancers fail to express differentiation-associated keratins, indicative of their undifferentiated state.

Utilizing in situ hybridization techniques and specific probes, filaggrin production is being characterized in skin from normal persons and from a variety of disorders of keratinization.

Project DescriptionMajor Findings:

A project studying keratin gene expression in skin cancer and cutaneous disorders of keratinization has been initiated. The study employs molecular hybridization probes that are specific for transcripts of individual keratin genes. The transcripts are localized at the cellular level within frozen sections by in situ hybridization with 35-S labeled RNA probes. Diseases studied to date include Darier's disease, psoriasis, lamellar ichthyosis, basal cell carcinoma and squamous cell carcinoma. Preliminary findings suggest that, in contrast to normal epidermis where the expression of proliferation-associated keratin genes is suppressed after cells migrate from the basement membrane, hyperproliferative disorders of the epidermis exhibit inappropriate expression of proliferation-associated keratin genes in the more superficial layers of the epidermis. In addition, skin cancers fail to express differentiation-associated keratins, indicative of their undifferentiated state. Recent experiments on normal skin indicate a sequential expression of differentiation-associated keratins (K1, K10).

Utilizing in situ hybridization techniques and specific probes, filaggrin production is being characterized in skin from normal persons and from a variety of patients with disorders of keratinization.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03638-20 D

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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

OTHER: V. A. Bohr, M.D., Senior Investigator, LMPH, DCT, NCI  
 M. Dizdaroglu, Ph.D., Research Chemist, NIST  
 A. J. Fornace, Jr., M.D., Senior Investigator, RO, NCI  
 K. H. Kraemer, M.D., Senior Investigator, LMC, DCE, NCI  
 L. Seguin, Ph.D., Consultant, D., DCBD, NCI  
 R. E. Tarone, Ph.D., Mathematical Statistician, BB, DCE, NCI

## COOPERATING UNITS (if any)

Laboratory of Molecular Pharmacology, DCT, NCI; Laboratory of Molecular  
 Carcinogenesis, DCE, NCI; Radiation Oncology Branch, DCT, NCI; Biostatistics  
 Branch, DCCP, NCI

## LAB/BRANCH

Dermatology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.8

## PROFESSIONAL:

1.8

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

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

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

Studies in this laboratory are designed to elucidate the role of DNA repair processes in carcinogenesis and in normal and abnormal aging and in neurodegeneration. Most studies have been conducted with cells from patients with xeroderma pigmentosum (XP), who have defective DNA repair plus multiple cutaneous malignancies and premature aging of sun-exposed skin and of the nervous system. Cells from patients with primary neuronal, muscular, and retinal degenerations are also being studied. These diseases include ataxia telangiectasia, Alzheimer disease, Parkinson disease, Huntington disease, Duchenne muscular dystrophy, retinitis pigmentosa, Friedreich ataxia, and Cockayne syndrome (CS). These studies are designed to elucidate the pathogenesis of these disorders. We assess the biological effectiveness of DNA repair by 1) in vitro assays of cell survival after treatment of the cells with DNA-damaging agents; 2) analysis of chromosome and chromatid aberrations in cells treated with DNA-damaging agents; and 3) transfection studies using irradiated plasmids. We search for DNA damage by 1) extracting the DNA and having it subjected to analysis by capillary gas chromatography-mass spectrometry; 2) studying unscheduled DNA synthesis induced in the overall genome of cultured cells by chemical carcinogens; and 3) studying DNA repair within an active gene. We search in patient cells for abnormalities in DNA-repair genes and in their DNA-damage-inducible transcripts using cDNA probes in Southern and Northern blot hybridization studies.

## Project Description

### Major Findings:

In collaboration with Dr. Vilhem A. Bohr and Dr. Michele K. Evans, we are studying the repair of ultraviolet radiation (UV)-induced cyclobutane pyrimidine dimers in an active housekeeping gene (the dihydrofolate reductase [DHFR] gene) as opposed to the conventional study of repair in the genome overall. In this procedure isolated DNA from fibroblasts is restricted, separated from replicated DNA, nicked at dimers with T-4 endonuclease V, electrophoresed in alkaline agarose, and subjected to Southern hybridization. We have studied normal and XP group A, C, and F fibroblast strains. We have found all the XP strains to have defective removal of dimers from the DHFR gene. The cells' relative capacity to repair the gene is the same as their relative capacity to perform UV-induced unscheduled DNA synthesis in the genome overall and does not correlate with the cells' relative ability to survive the lethal effects of UV. These studies show that removal of dimers from an active gene is not required for high survival, since the relatively UV-resistant group F strain showed virtually no dimer removal. Our results indicate that some lesions(s) other than dimers is likely to be important in UV lethality. We plan to adapt the active-gene-repair assay to monitor the repair of nondimer lesions induced by UV, for example, the 6-4 photoproduct. We plan also to study active-gene-repair in Cockayne syndrome cells in an attempt to confirm reports of others that in Cockayne syndrome there is defective DNA repair in active genes but not in the genome overall.

In collaboration with Dr. Kenneth H. Kraemer, we are continuing to transfect human lymphoblastoid cell lines with a plasmid (pRSVcat) which contains the bacterial cat gene coding for chloramphenicol acetyltransferase. The plasmid is previously irradiated with different doses of 254-nm UV. Then, in order to evaluate the repair of dimer and nondimer photoproducts, aliquots containing the irradiated plasmid are treated with photoreactivating enzyme (E. coli photolyase) and light, a treatment which monomerizes the UV-induced cyclobutane-type of pyrimidine dimer and leaves only nondimer photoproducts. We now have evidence that at least some Cockayne syndrome fibroblast strains, like XP fibroblast strains, are unable to repair not only pyrimidine dimers but also nondimer photoproducts induced in the plasmid by UV.

With Dr. Albert J. Fornace, Jr., we have studied DNA from normal and UV-sensitive cells in Southern analysis using a total of 9 different cDNA probes which encode UV-inducible transcripts in mammalian cells. One of our goals is to find evidence for defects in the relevant gene in certain UV-sensitive cell strains. We have found no significant complications due to polymorphisms. We have not yet found an abnormality in any of the 9 relevant genes tested for so far. We are also studying the expression of 2 transcripts which are induced in normal human cells and in radiosensitive ataxia telangiectasia cells by several DNA-damaging agents including X rays and growth arrest. Since ataxia telangiectasia cells have abnormalities in arrest of DNA synthesis after treatment with ionizing radiation, we should be able to determine whether or



not these DNA-damage-inducible genes are abnormally regulated in the ataxia telangiectasia cells.

We have concluded our collaboration with John Nove and Dr. John B. Little (Harvard University School of Public Health) into the post-X-ray survival capacity of fibroblast strains from patients with the neurodegenerative diseases Friedreich ataxia and Alzheimer disease. We found the Friedreich ataxia cells to have normal sensitivity, while the Alzheimer disease cells appeared to be slightly hypersensitive. These results are compatible with the possibility that Alzheimer disease cells have defective repair of DNA damage induced by ionizing radiation.

We have performed autoradiographic studies of methylmethane sulfonate (MMS)-induced unscheduled DNA synthesis in fibroblasts from patients with amyotrophic lateral sclerosis or group D xeroderma pigmentosum. These studies are being performed in an attempt to confirm published reports of others that such fibroblasts are deficient in repairing MMS-induced DNA damage. Our preliminary results indicate no evidence for such an abnormality in either cell type.

All XP patients have abnormally increased frequencies of UV-induced skin cancers. It is known that nucleotide excision-repair-deficient XP patients have abnormally high levels of UV-induced chromosomal aberrations. We are continuing our studies to determine the levels of such aberrations in cells from XP variant patients (who have normal levels of nucleotide excision repair). These studies may indicate whether or not abnormal levels of UV-induced chromosomal aberrations are causally related to the development of sunlight-induced skin cancer in XP patients. Our results indicate that XP variant cells have only a minimal, if any, abnormally high level of UV-induced chromosomal aberrations.

We are continuing our studies with Dr. Miral Dizdaroglu using capillary gas chromatography-mass spectrometry to detect various types of damage induced in DNA by free radicals and by ionizing radiation. We are concentrating on determining the level of such damage which may normally be present in unirradiated cells in tissue culture and in vivo. Various types of such damage appear to be present in all preparations of DNA and in many commercial preparations of the DNA bases (e.g., thymine, adenine and guanine) we have studied. We have preliminary evidence that such damage is not caused by the derivatization process used in our assay, and we are now determining whether or not the hydrolysis to which we subject DNA (to free the bases and nucleosides we analyze) produces the damage.

Publications:

Robbins JH. Defective DNA repair in xeroderma pigmentosum and other neurologic diseases, *Curr Opin Neurol Neurosurg* 1988;1:1077-1083.

Robbins JH. A childhood neurodegeneration due to defective DNA repair: a novel concept of disease based on studies of xeroderma pigmentosum. *J Child Neurol* 1989;4:143-146.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03656-16 D

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments

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

P.I.: P.M. Steinert, Biologist, Dermatology Branch, NCI

OTHER: see next page

## COOPERATING UNITS (if any)

Lab of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI; Laboratory of Physical Biology, NIAMS; Laboratory of Biochemistry, NIDR

## LAB/BRANCH

Dermatology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

7.5

## PROFESSIONAL:

5.5

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

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

B

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

The biosynthesis, structure and function of the principal differentiation products of human and mouse epidermis are being studied. Keratin subunits polymerize in vitro into native-type intermediate filaments. Details of their structure are being investigated by use of: solid state NMR on isotopically-labeled filaments; transmission and scanning transmission electron microscopy of intact filaments or subfilamentous forms; optical diffraction and image analysis procedures; and limited proteolysis experiments to ascertain alignment of constituent subunits. Model structures generated by these methods are being computationally tested for compatibility with other physico-chemical data and amino acid sequence information on individual subunits. PCR-generated probes to type I and type II keratins are being used to characterize the number, organization and complexity of their genes. The expression of the human keratin genes 1 and 10 are being studied by the production of transgenic mice produced from various constructs of these genes. cDNA clones are being used to isolate and characterize the genes for human filaggrin, which appear to be initially expressed as a very large polypeptide precursor. Clones encoding a major cell envelop protein have been isolated and are being sequenced. Constructs of keratins, filaggrin and the cell envelop protein clones have been assembled with pGEM vectors for use in *in situ* hybridization experiments in order to study the expression of these proteins in epidermal keratinizing disorders.

Other Professional Personnel:

S.-Q. Gan, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI  
 W.W. Idler, B.S., Chemist, Dermatology Branch, DCBD, NCI  
 J. Mack, Ph.D., Staff Fellow, Dermatology Branch, DCBD, NCI  
 J. Compton, Ph.D., Jackson Laboratories, Bar Harbor, ME  
 J. Chung, Ph.D., Senior Investigator, Laboratory of Biochemistry, NIDR  
 R.D. Goldman, Ph.D., Professor, Dept. Antmy & Cell Biol., Northwestern Univ.  
 D. Hohl, M.D., Guest Researcher, Dept. Cell Biology, Baylor College of  
 Medicine, Houston, TX  
 S. Lessin, M.D., Assistant Professor, Dermatology Department and Wistar  
 Institute, Philadelphia, PA.  
 L.J. McKinley-Grant, M.D., Guest Researcher, Dermatology Branch, DCBD, NCI  
 D.A.D. Parry, Ph.D., Professor, Dept. of Physics and Biophysics, MasseyUniv.,  
 Palmerston North, New Zealand  
 D.R. Roop, Ph.D., Associate Professor, Dept. Cell Biology, Baylor College of  
 Medicine, Houston, TX  
 A.C. Steven, Ph.D., Visiting Scientist, Lab. of Phys. Biol., NIAMS  
 J. Wall, Ph.D., Professor, Department Biology, Brookhaven National Laboratory,  
 Upton, NY  
 S.H. Yuspa, M.D., Branch Chief, Lab. of Cellular Carcinogenesis and  
 Tumor Promotion, DCE, NCI

Project DescriptionMajor Findings:

The availability of complete amino acid sequences of several keratin chains has now led us to propose more advanced models of intermediate filament structure. Even though filaments are polymorphic, a major structural form consists of 8 4-chain molecules, arranged in an antiparallel mode. The structure of the 4-chain molecule is being examined by scanning transmission electron microscopy (with Dr. Wall) using platinum-clathrates complexed onto cystine residues. In collaboration with Drs. A.C. Steven and David Parry, we have constructed "surface-lattice" models of filament structure in which the rod domains of the protein chains form the basic filament core, and the variable end domains occupy peripheral positions. Limited proteolytic cleavage experiments have provided direct evidence in support of these structural models and of the precise alignment of the neighboring rod domains. In addition, scanning transmission electron microscopy is being used to explore the mass, structure and association of 4-chain units in an attempt to describe intermediate levels of filament substructure. However, the structural organization of the end domains is still uncertain. In the case of the keratins, we propose that their glycine-serine-rich sequences form omega-loop structures and interact with other cellular components including filaggrin and the cell envelop by H-bonding and disulfide cross-links. In collaboration with Dr. James Mack, these ideas are being tested by use of solid-state NMR of keratin filaments isotopically labeled with glycine (to study end domains) and leucine (to study the rod domain). Our data suggests the glycine-rich domains have little or no structural order, whereas the rod-domains are considerably more ordered.



We are using NMR techniques to study the nature of the molecular interaction of keratin filaments and filaggrin, which is an important filament associated protein in the epidermis. Keratin filaments isotopically labeled with glycine (for end domains) and glutamic acid or lysine (for rod domains) are complexed with filaggrin. Changes in the molecular motions of the filaments will provide information on their mode of interaction with filaggrin. Preliminary data suggests the molecular motions of the glycine regions are unchanged, implying indirectly that filaggrin interacts with the rod domains. Experiments in progress with lysine -or glutamic acid- labeled filaments should provide more direct evidence for this mechanism.

Many cDNA clones to human filaggrin have now been isolated and sequenced. The human filaggrin gene encodes a polyprotein containing a large number of filaggrin repeats arranged in tandem. Sequencing by Dr. Gan has shown that these repeats are highly polymorphic but conserved in size. Also, the gene from different individuals contain differing numbers of repeats. In collaboration with Dr. Lessin, we have localized the human gene to chromosome 1q21 region. Attempts are now underway to isolate the human gene in order to define regulatory sequence regions by use of CAT assays and eventually production of transgenic mice bearing the human gene.

Dr. Hohl has isolated, cloned and sequenced the gene for the major human cornified cell envelop proteins, named loricrin. Fragments of this gene are now being subcloned into CAT vectors to define regulatory regions. In collaboration with Dr. Dennis Roop, the clones bearing the complete gene will be used for the production of transgenic mice. In collaboration with Dr. Ron Chung, we have begun to isolate and characterize cDNA clones for epidermal transglutaminase, the enzyme which cross-links the loricrin to the cell envelop. In addition, we have synthesized peptides corresponding to loricrin sequences for in vitro transglutaminase assays, and <sup>3</sup>H-lysine-labeled cross-linked peptides of loricrin from labeled foreskins will be characterized to define the structural organization of loricrin in the cell envelop.

A major project to determine the structure, complexity, linkage and evolution of keratin genes has been initiated. In collaboration with Dr. Stuart Lessin, we have found that type II keratins seem to be linked in one cluster in the general region of 12q11-12 and type I keratins are clustered on 17q12-21. Using keratin probes generated by PRC reaction to known sequence, we are employing pulse field electrophoresis to further map the linkage of the keratin genes. In related experiments in collaboration with Drs. Compton and Roop, the human keratin 1 and 10 genes have been used to generate transgenic mice with the aim to study their regulation. Also mutant versions of these genes will be used to explore the possibility of generating disorders of keratinization due to the expression of the mutant phenotype.

In collaboration with Drs. Hohl, Roop and McKinley-Grant, in situ hybridization techniques are being used to study the expression of filaggrin, loricrin and keratins 1 and 10 in disorders of keratinization.

Publications:

McKinley-Grant LJ, Idler WW, Bernstein IA, Parry DAD, Cannizzaro L, Croce CM, Huebner K, Lessin SR, Steinert PM: Characterization of a cDNA clone encoding human filaggrin and localization of the gene to chromosome region 1q21, Proc Natl Acad Sci USA 1989 (in press).

Steven AC, Mack JW, Trus BL, Bisher ME, Steinert PM: Structure and assembly of intermediate filaments: multi-faceted, myosin-like cytoskeletal polymers. In: Cytoskeletal and Extracellular Proteins, Structure, Interactions and Assembly, ed. by Aebi UC, Engel J, the 2nd EBSA Symposium, pp 15-26, 1989.

Yuspa SH, Kilkenny AE, Steinert PM and Roop DR: Expression of epidermal differentiation markers is regulated by specific extracellular calcium concentrations, J Cell Biol 1989 (in press).

Steinert PM, Roop DR: The structure, complexity and evolution of intermediate filament genes. In: The Cellular and Molecular Biology of Intermediate Filaments, ed. by Goldman RD, Steinert PM, Plenum, NY 1989 (in press).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03668-01 D

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Molecular and Cell Biology of Immunoreactive Molecules in the Skin

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

P.I.: S. Wright Caughman, M.D., Dermatology Branch, DCBD, NCI

OTHER: Klaus Degitz, M.D., Guest Researcher, Dermatology Branch, DCBD, NCI  
Lian-Jie Li, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI

## COOPERATING UNITS (if any)

Cell Biology and Metabolism Branch, NICHD  
Department of Dermatology, Emory University Medical Center, Atlanta, GA

## LAB/BRANCH

Dermatology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 10892

## TOTAL MAN-YEARS:

2.42

## PROFESSIONAL:

2.42

## OTHER:

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 purpose of my laboratory is to elucidate the molecular mechanisms regulating the expression of immunoreactive molecules in the skin. ICAM-1, or intercellular adhesion molecule-1, is a cell surface protein that is the major ligand for LFA-1, or lymphocyte function associated antigen-1, an anchoring receptor on all T cells whose interaction with ligand is critical for proper T-cell function. Cutaneous T-cell inflammatory and neoplastic diseases may well depend upon the regulation of ICAM-1 expression by various cells in the skin. We have established that human keratinocytes (HK) in culture do not normally express ICAM-1, and that exposure of HK to human interferon-gamma (IFN) or tumor necrosis factor-alpha (TNF), two biologic response modifiers (BRM), results in the rapid induction of ICAM-1 mRNA expression, protein biosynthesis, and surface expression. We have demonstrated that human dermal microvascular endothelial cells (HDMEC) constitutively express ICAM-1, that this expression can be rapidly upregulated at the level of mRNA and surface expression by exposure to human interleukin-1 (IL-1), and that specific T-cell binding to HDMEC in vitro is dependent upon ICAM-1 expression by HDMEC and can be upregulated as well by factors which increase ICAM-1 expression. We have established that ICAM-1 expression in HK, and HDMEC is regulated at the level of gene transcription and we have isolated a 19 kb genomic clone containing portions, if not all, of the ICAM-1 gene. Mapping and sequencing of the 5' flanking regulatory region of this genomic clone of ICAM-1 has been partially completed. Through a continuing collaboration, we have also continued our analysis of the translational regulation of human ferritin expression. Ten different reporter gene constructs containing various combinations of functional and nonfunctional iron-responsive elements in their translational regulatory regions have been produced, their sequences confirmed, and stably expressing transfectants established.

## Project Description

### Major Findings:

Human keratinocytes (HK) do not normally express ICAM-1 *in vitro*, but can be induced to express this T-cell ligand by various biologic response modifiers, including IFN-gamma and TNF-alpha.

Exposure of HK to IFN-gamma results in the rapid induction of ICAM-1 mRNA, ICAM-1 protein biosynthesis, and ICAM-1 surface expression. This rapid induction of expression is completely inhibited by pre-exposure to actinomycin-D, indicating that expression is regulated at the level of transcription.

ICAM-1 expression induced by IFN-gamma exhibits a dose- and time-dependent response with regard to mRNA induction, protein biosynthesis, and surface expression. While induction is detectable with as little as 1 unit IFN-gamma per ml of culture media for 1 hour, optimal expression occurs at 25 U/ml after 12 hours incubation.

Induction of ICAM-1 expression in HK by IFN-gamma is not inhibited by pre-exposure to transforming growth factor-beta, a substance previously implicated in the inhibition of ICAM-1 expression, either at the level of surface expression or mRNA induction, even at sub-optimal dosages and time exposures of IFN-gamma.

Human dermal microvascular cells (HDMEC) constitutively express ICAM-1 at the level of mRNA and surface protein. Upregulation of ICAM-1 mRNA and surface expression as well as upregulation of ICAM-1 dependent T-cell binding to HDMEC is achieved by exposure of HDMEC to IL-1 in a dose- and time-dependent manner.

A 19 kb genomic clone which hybridizes with a 5' fragment of a human ICAM-1 cDNA has been isolated from a human T-cell genomic library. In an attempt to identify the transcriptional regulatory regions and possible trans-acting factors affecting expression of the ICAM-1 gene, a 5.5 and a 2.2 kb fragment which hybridize with the 5' fragment of the cDNA clone have been identified, subcloned, and partially mapped and sequenced.

The iron-responsive translational regulation of ferritin protein biosynthesis is dependent upon an oligonucleotide sequence appearing in the 5' untranslated region of the ferritin mRNA. This sequence, or iron-responsive element (IRE), has been previously identified and critical bases determined. We have produced constructs using varying numbers of synthetic oligonucleotides encoding functional and nonfunctional IRE's in the 5' untranslated regions of reporter genes. We have confirmed the number, order and sequence of these differing multiple IRE constructs and have established stably expressing transformants of each in murine B6 cells. These transformants will be utilized to analyze the effects of number, functionality, and relative



location of IRE's in relation to mRNA levels, indicator gene biosynthetic rates, and relative translatability in response to changes in iron concentration.

Publications:

Casey JL, Hentze MW, Koeller DM, Caughman SW, Rouault TA, Klausner RD, Harford JB. Iron-responsive elements: regulatory RNA sequences control mRNA levels and translation, *Science* 1988;240:924-928.

Rouault TA, Hentze MW, Caughman SW, Harford JB, Klausner RD. Binding of a cytosolic protein to the iron-responsive element of human ferritin messenger RNA, *Science* 1988;241:1207-1210.

Hentze MW, Caughman SW, Casey JL, Koeller DM, Rouault TA, Harford JB, Klausner RD. A model for the structure and functions of iron-responsive elements, *Gene* 1988;72:201-208.

Caughman SW, Hentze MW, Rouault TA, Harford JB, Klausner RD. The iron-responsive element is the single element responsible for iron-dependent translational regulation of ferritin biosynthesis: evidence for function as the binding site for a translational repressor. *J Biol Chem* 1988;263:19048-19052.

Rouault TA, Hentze MW, Caughman SW, Casey JL, Koeller DM, Harford JB, Klausner RD. Post-transcriptional regulation of ferritin and transferrin receptor expression: role of iron-responsive elements. In: Hamer DH, Winge DR, eds. *Metal Ion Homeostasis*. New York, Alan R. Liss, 1989;109-115.

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

The clinical research program of the Metabolism Branch is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations are carried out in patients with immunodeficiency diseases that are associated with a high incidence of neoplasia, as well as in patients with malignancy, especially T- and B-cell leukemias. These studies are directed toward defining the factors involved in the control of the human immune response. Major efforts in this area are directed toward: 1) studies of the arrangement of the immunoglobulin genes and T-cell antigen-specific receptor genes and the rearrangements and deletions of these genes that are involved in the control of the synthesis of immunoglobulins and T-cell receptors; one focus in this area is the study of novel transforming genes that translocate into the T-cell receptor locus in T-cell tumors; 2) the characterization of transacting regulatory factors that mediate lymphocyte specific gene transcription; the scientific focus of this area is the purification of the transactivating factors, the cloning of the genes encoding these factors and the definition of their mode of action at a molecular level; 3) somatic gene therapy for human genetic immunodeficiency diseases; 4) genetic control of the immune response; one emphasis of this area is the development of a novel method for predicting molecular structures recognized by T-cells and the applications of this algorithm to the development of vaccines aimed at preventing AIDS and malaria; 5) identification, purification, and molecular genetic analysis of the multichain interleukin-2 receptor on normal and malignant lymphocytes; one emphasis of this area is the development of different forms of IL-2 receptor directed therapy; 6) analysis of action of immunoregulatory cells including helper T cells, suppressor T cells, and macrophages that regulate antibody responses and on studies of disorders of these immunoregulatory cell interactions and of leukemias of these immunoregulatory cells; and 7) isolation and characterization of biological response modifiers and oligosaccharides that regulate the human immune response. The second major goal of the Metabolism Branch is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Both patients with neoplastic diseases as well as those with nonneoplastic disorders that facilitate the development of techniques for the study of cell membranes, homeostatic mechanisms, and metabolic derangements of biochemical control mechanisms are being investigated. Within this area, one special emphasis is placed on the normal growth factors especially insulin-like growth factors that play a role in the hormonal control of normal and malignant growth. An additional focus is on the regulatory role on intermediary metabolism played by proline and its metabolite pyrroline-5-carboxylate.

MOLECULAR ANALYSIS OF TRANSLOCATIONS OF TRANSFORMING GENES TO  
THE LOCUS OF T-CELL ANTIGEN RECEPTOR GENES

Previously Dr. Thomas Waldmann and Dr. Stanley Korsmeyer introduced the analysis of immunoglobulin and T-cell receptor gene rearrangements to define the

lineage (T or B cell) of cells lacking conventional markers, to establish whether abnormal lymphocytic populations are polyclonal, oligoclonal or monoclonal, and to broaden the scientific basis for the diagnosis and monitoring of the therapy of lymphoid neoplasia. More recently, Dr. Waldmann has exploited illegitimate chromosomal translocations in T cell leukemia involving the T cell receptor genes to clone elements involved in the regulation of normal and malignant cell proliferation. Dr. Glenn Begley, in collaboration with Dr. Ilan Kirsch, has identified a putative transforming gene which they call SCL-1 that is expressed in hematopoietic tissues. The gene was identified through the molecular cloning of a translocation between chromosomes 1 and 14;t(1:14)(p33;q11) that occurred in a human stem cell leukemia that was able to differentiate into myeloid and lymphoid lineages. As a consequence of the translocation, a novel fusion transcript was generated between the sequences on chromosome 1 and chromosome 14. The gene of chromosome 1 manifested a DNA binding motif shared with the *Drosophila* daughterless gene and the myc and MyoD proteins. This gene was expressed in fetal liver and normal bone marrow and may be important for hematopoietic stem cell development and oncogenesis.

#### MOLECULAR ANALYSIS OF TRANS-ACTING FACTORS THAT MEDIATE LYMPHOID SPECIFIC GENE TRANSCRIPTION

As an approach to understanding development in the B lymphocyte lineage, Dr. Louis Staudt has characterized trans-acting factors that mediate lymphoid-specific gene transcription. As a model he has studied the lymphoid-specific transcription of immunoglobulin (Ig) genes. The promoters of all Ig variable genes and the Ig heavy chain enhancer contain a critical octamer DNA motif, ATTTGCAT. He demonstrated that this motif, together with a TATA motif, is necessary and sufficient for lymphoid-specific promoter activity. He then assayed nuclear extracts of lymphoid and non-lymphoid cell lines for proteins which specifically bound to the octamer motif and detected two major species: a ubiquitous octamer binding protein (Oct-1) and a lymphoid-specific octamer binding protein (Oct-2). Oct-2 functioned as a transcription factor in vitro using immunoglobulin promoter templates. He cloned the human Oct-2 structural gene by screening a phage  $\lambda$ gt11 expression library with a radiolabeled DNA probe containing the octamer motif. The cloned gene encodes an octamer-specific DNA binding protein which is itself lymphoid-restricted in expression.

By sequencing the Oct-2 gene Dr. Staudt identified a 60 amino acid domain that is homologous to the "homeo box" domain of proteins which are important for cell type determination and development in lower eukaryotes. This domain of the Oct-2 protein was shown to be required for DNA binding by site directed mutagenesis. This represents the first demonstration that a mammalian gene containing a homeo box domain is a sequence-specific DNA binding protein. The presence of the homeo box in the Oct-2 gene lends support to the notion that this gene is important for lymphoid development. Full length Oct-2 cDNA clones revealed additional regions of homology with other transcription factors. One highly conserved 75 amino acid domain, the "POU" box, is shared by a subset of homeo box proteins that includes a pituitary-specific human transcription factor, Pit-1. In addition, Oct-2 has a "leucine zipper" domain which it shares with the nuclear oncogenes myc, fos and jun and with other transcription factors. The leucine zipper may mediate the interaction of Oct-2 with other regulatory proteins. Alternative splicing of the Oct-2 gene generates a variety of distinct Oct-2 proteins, one of which lacks the leucine zipper and thus might be functionally unique.



Recent evidence obtained by Dr. Staudt indicates that the octamer motif may control the expression of other lymphoid-specific genes besides Ig, notably the Bcl-2 proto-oncogene involved in the pathogenesis of human follicular lymphomas. Using the cloned Oct-2 gene, Dr. Staudt should be able to determine the range of genes which are expressed exclusively in lymphocytes under the transcriptional control of the octamer motif. Oct-2 is present in some T cells and is inducible in this lineage by T cell activation signals suggesting that Oct-2 may be involved in the expression of T cell activation genes.

#### SOMATIC GENE THERAPY FOR HUMAN GENETIC DISEASE

Dr. Michael Blaese has continued his research program to develop gene transfer for the therapy of human disease. He and his collaborators, Steven A. Rosenberg and W. French Anderson, have now performed the first authorized clinical experiment in which a foreign gene has been introduced into humans. A recombinant murine retroviral vector containing the bacterial gene encoding Neomycin resistance (neoR) was used to label tumor infiltrating lymphocytes (TIL) used in the treatment of patients with malignant melanoma. These neoR expressing TIL were reinfused into the patients to permit Dr. Blaese and his associates to study the survival and trafficking patterns of TIL in an attempt to determine whether they can identify any characteristics which correlate with the clinical efficacy of the TIL in inducing tumor remission. The next phase of these studies will involve the insertion of genes encoding various cytokines into TIL in an attempt to improve their tumoricidal activity for a variety of tumors. Dr. Blaese's laboratory has prepared vector constructs with the alpha and gamma interferon genes as well as genes for TNF and IL-1 $\beta$ . These vector preparations are currently being characterized for their ability to produce these cytokines in both lymphoid and non-lymphoid cells. Major studies of the biological effects of cells constitutively secreting these molecules are also underway as are studies of the potential utility of using lymphocytes as cellular vehicles for gene therapy of non-malignant diseases such as ADA deficiency. T lymphocytes from SCID patients have been grown to high numbers using anti-CD3 and rIL-2 stimulation and the human ADA gene has been successfully introduced into these cells. A clinical protocol for the treatment of ADA(-)SCID with gene-treated cultured T cells is now being prepared for submission to the Recombinant DNA Advisory Committee.

Dr. Blaese has also continued his work with the compound Succinylacetone (SA) which he, Richard Hess and Donald Tschudy discovered to have potent immunosuppressive properties. The most recent work with this drug has involved solid organ transplantation studies in both rats and primates in collaboration with Dr. Christopher Stone of the NHLBI. SA has now been demonstrated to greatly prolong skin allograft survival in rats and to permit indefinite survival of totally mismatched cardiac allografts in rats. Studies of heterotopic cardiac allografts and rhesus and cynomolgus monkeys have also shown very significant graft prolongation in these animals although the grafts have only been in place for six weeks as of the date of this report (normal rejection is within 8 $\pm$ 2 days). Toxicity in these primates has been low.

#### THE GENETIC CONTROL OF THE IMMUNE RESPONSE: APPLICATION TO THE DESIGN OF SYNTHETIC VACCINES

Dr. Jay Berzofsky has been studying the mechanisms by which T cells recognize antigens on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, the factors that determine which antigenic



structures are more likely to be recognized, and the application of these principles to the design of synthetic vaccines for AIDS and malaria. T cells recognize antigen after it has been proteolytically processed into fragments which then associate with MHC molecules. As a first step toward prediction of the sites of cleavage, Dr. Berzofsky has analyzed the proteases responsible for processing myoglobin to present three different determinants to different T-cell clones. The same protease, an intracellular thiol protease such as cathepsin B, was both necessary and sufficient to process myoglobin to present all three determinants. Second, different MHC molecules preferentially present different peptides from the same protein. To determine the role of residues of the MHC molecule more directly, Dr. Berzofsky studied a series of site-directed mutants containing single amino acid substitutions, or groups of substitutions, that correspond to polymorphic differences between two alleles of this class II MHC molecule. Mutations at certain positions could differentially affect presentation of the same antigenic determinant to different T-cell clones. This result suggests that different T cells can see the same determinant in different positions or conformations in the same MHC molecule. Third, Dr. Berzofsky examined structural features intrinsic to the antigenic determinant, and confirmed on a much larger database that peptides that can fold as amphipathic alpha helices are statistically more frequent among immunodominant T-cell determinants than in protein sequences in general. In addition to sites that are immunodominant with class II MHC molecules (71%,  $p < 0.003$ ), he found that 10/11 sites seen with class I MHC molecules also have this property, indicating that the same chemical principles probably apply to recognition with class I MHC molecules as well. These correlations may be valuable in searching for immunodominant determinants for vaccine development.

Dr. Berzofsky applied these results to the design of synthetic vaccines for malaria and AIDS. In the malaria case he had previously found two immunodominant sites for helper T cells of both mice and humans. He found that T cells from some humans from a malaria endemic-area of Africa could distinguish the variants of these sites from different isolates of *P. falciparum*, so that the mutations are not immunologically silent. On the other hand, evidence was obtained for crossactivity among the variants of both sites, so that it should be possible to develop a vaccine that may be useful for many strains of *P. falciparum*. In the case of AIDS, Dr. Berzofsky has identified 6 multideterminant regions of the envelope protein that contain overlapping helper T-cell antigenic determinants seen by mice of different MHC types, so that each region can be seen by most MHC types, allowing circumvention of MHC restriction problems. Four of the peptides were studied in HIV-infected humans. He found that about 80% of humans early in HIV infection lose the ability to respond in a T-cell proliferative response to soluble protein antigens, but about 50% retain a T-cell response to the same antigens as measured by IL-2 production. This finding may be of prognostic significance. Each peptide elicited *in vitro* T-cell responses in cells from 50-67% of unrelated HLA-diverse donors who retained the ability to respond to control antigens, and 85-90% of such donors responded to at least one of the 4 peptides, so many peptides may not be required for an MHC-diverse population. Also, Dr. Berzofsky found that one of these peptides served as a very potent helper determinant when attached to a neutralizing antibody epitope to elicit neutralizing antibodies better than a protein carrier. Finally, because the CTL determinant he previously identified in the envelope protein is highly variable among HIV isolates, he examined the effect of this variability on T-cell recognition and found that the same site is a CTL determinant on several isolates of HIV. By studying substitutions at each position, he could identify residues that contribute to binding to class I MHC molecules, and one residue that seems critical for recognition by the TCR. Thus,

HIV can mutate residues that interact with MHC molecules. Because Dr. Berzofsky has found that sites on HIV proteins that are presented by class I MHC molecules are rare, he proposes that such mutations in critical sites that prevent binding to MHC molecules of an individual may allow the virus to become partially invisible to CTL of the person, escape control, and cause AIDS. The identification of these helper and CTL determinants, approaches to circumvent MHC restriction, and the understanding of virus variability, should prove useful in the design of vaccines.

#### THE MULTI-CHAIN IL-2 RECEPTOR: CHEMICAL CHARACTERIZATION, FUNCTION AND USE AS A TARGET FOR IMMUNOTHERAPY

The success of the critical regulatory and effector functions of T lymphocytes requires that these cells change from a resting to an activated state. Antigen-stimulated activation of resting T cells induces the synthesis of interleukin-2 as well as high affinity IL-2 receptors that are not expressed on resting T cells. Dr. Waldmann previously identified two peptides that bind IL-2, the 55 kD peptide reactive with the anti-Tac monoclonal, and a novel 70/75 kD (p75) peptide. Dr. Waldmann proposed a multichain model for the high affinity IL-2 receptor in which both the p55 Tac and the p75 IL-2 binding peptides are associated in a receptor complex. Recently Dr. Waldmann and his coworkers, using crosslinking, coprecipitation and fluorescence energy transfer techniques, have identified additional p22, p35, p40, p95 and p180 peptides that may be associated with the 55 kD Tac peptide in the multichain receptor.

One of Dr. Waldmann's most critical contributions was his recognition that the IL-2 receptor represents an extraordinarily versatile therapeutic target. He entered this area to exploit his observations that T cells in patients with certain lymphoid malignancies (e.g., adult T cell leukemia, cutaneous T cell lymphoma, hairy cell leukemia and Hodgkin's disease) and select autoimmune diseases (e.g., scleroderma, tropical spastic paraparesis and rheumatoid arthritis) as well as individuals rejecting allografts express the IL-2 receptors identified by the anti-Tac monoclonal antibody whereas normal resting cells and their precursors do not. He reasoned that agents that could eliminate Tac-expressing leukemic cells or activated T cells involved in other disease states could be effective therapies for these disorders yet would retain the Tac-negative mature normal T cells and their precursors. Dr. Waldmann and his coworkers have developed a wide variety of therapeutic reagents. Dr. Waldmann has effectively utilized the unmodified anti-Tac monoclonal antibody to block binding of IL-2 to its growth factor receptor and to lead to remissions lasting 1, 5 and over 8 months in the treatment of patients with adult T cell leukemia. This therapy that has not had toxic complications has also been used effectively in the prevention of early graft rejection in patients receiving donor renal allografts.

In order to improve the effectiveness of IL-2 receptor directed therapy, different approaches have been used to modify the antibody to augment its capacity as a cytotoxic agent. For example, in collaboration with Dr. Ira Pastan a genetically modified form of Pseudomonas exotoxin (PE40) which has been developed from which the first domain which is responsible for ubiquitous cell binding was deleted. This truncated toxin was conjugated to anti-Tac or IL-2 in order to provide an antibody or alternatively a lymphokine-mediated method of delivering the toxin directly to the surface of IL-2 receptor expressing cells. Anti-Tac (Fv) sequences encompassing the variable regions of the anti-Tac light- and heavy-chains were genetically coupled to the truncated Pseudomonas exotoxin sequence to generate a single co-linear protein with the specificity of the antibody. In addition, all

studies alternative cytotoxic agents were developed that could be conjugated to anti-Tac that were effective when bound to the surface of Tac-expressing cells. Specifically, in conjunction with Dr. Otto Gansow, Dr. Waldmann showed using in vitro assays and in vivo cardiac allograft models that  $^{212}\text{Bi}$ , an alpha-emitting or  $^{90}\text{Y}$  a beta-emitting radionuclide conjugated to anti-Tac by use of bifunctional chelates were effective and specific immunocytotoxic agents for the elimination of Tac-expressing cells. For example, the administration to subhuman primates of  $^{90}\text{Y}$  yttrium anti-Tac led to a marked prolongation of the survival of both cardiac xenografts and allografts when compared to the survival in untreated animals or in animals treated with unmodified anti-Tac. The use of mouse anti-Tac is hindered by the host immune response to the murine antibody and by the lack of effector cytotoxic capacity of the monoclonal. Therefore in collaboration with Dr. Cary Queen, Dr. Waldmann and his coworkers have constructed a "humanized" anti-Tac antibody by placing the complementary determining regions (CDRs) of the murine anti-Tac antibody into the human variable region framework and  $\gamma_1$  constant regions. In addition, a molecular model of the murine anti-Tac was used to identify the amino acids which although outside of the CDRs had a high probability of interacting with either the CDRs or the antigen surface. Such residues could have an effect on antigen binding and thus were retained in the constructed antibody.

Over the past year, hyperchimeric "humanized" anti-Tac antibodies have been generated in which the molecule is entirely human IgG1 kappa except for the small hypervariable segments of the complementarily determining regions retained from the mouse antibody. It is hoped that these "humanized" antibodies will be minimally immunogenic. Furthermore, they add the new functional activity of antibody dependent cellular cytotoxicity that was absent in the parent mouse anti-Tac while maintaining the important functions of high affinity binding and T-cell receptor blockade. Clinical trials will be initiated to define the efficacy of each of these agents in the treatment of patients. Dr. Waldmann's studies on the IL-2/IL-2 receptor system open the possibility for a major advance toward more specific, controlled immunosuppressant strategies. Thus the clinical application of highly potent anti-IL-2 receptor-directed agents represents a new therapeutic perspective for certain neoplastic diseases, autoimmune disorders and for the prevention of allograft rejection.

Dr. David Nelson has identified a soluble form of the p55 component (Tac protein) of the human interleukin-2 receptor in the supernatants of activated T-cells, B-cells, and monocytes in vitro and in the serum of normal individuals in vivo. Elevated levels of soluble Tac protein were found in the sera of patients with a variety of lymphoreticular neoplastic conditions including the adult T-cell leukemia (ATL), hairy cell leukemia (HCL), Hodgkin's and non-Hodgkin's lymphomas, and in patients with the acquired immunodeficiency syndrome (AIDS). In ATL and HCL patients, the serum level of Tac protein was indicative of tumor burden and favorable responses to therapy were associated with reductions in the serum level of Tac protein. Elevations of Tac protein in serum were also observed associated with allograft rejection episodes in patients with liver and heart-lung transplants. Patients with autoimmune diseases also had elevated levels of Tac protein in serum and joint fluids. In rheumatoid arthritis patients, the level of soluble Tac protein correlated with disease activity. Elevated levels of soluble Tac protein were also found in the sera of patients with neurologic diseases including multiple sclerosis and schizophrenia. The measurement of soluble Tac protein in various body fluids is useful in monitoring certain neoplastic and immune-mediated events in vivo.



To gain further insight into the regulation of the human immune response molecular genetic studies of a kindred with X-linked hypogammaglobulinemia and isolated growth hormone deficiency were initiated. Mapping studies by restriction fragment length polymorphism have shown that this disease is due to a gene distinct from that causing the more common form of X-linked agammaglobulinemia or Bruton's disease.

#### ISOLATION AND CHARACTERIZATION OF BIOLOGICAL RESPONSE MODIFIERS AND OLIGOSACCHARIDES THAT REGULATE THE HUMAN IMMUNE RESPONSE

Dr. Andrew Muchmore has continued his interest in the role of carbohydrate recognition in regulation of the human immune response. He has demonstrated that mannose oligosaccharides inhibit T cell responsiveness, enhance PGE2 synthesis, induce IL-1 and TNF production, and directly activate the hexose monophosphate shunt. In vitro models demonstrate that rIL-1, rTNF, and rIL-2 also bind to unique high mannose structures. This interaction occurs optimally at an acidic pH and his in vitro and in vivo data support the hypothesis that the endosome lysosome compartment is a major site of action for these cytokines. Furthermore, the carbohydrate binding region of these cytokines appears to be critical for their action. Dr. Muchmore's data also support a role for uromodulin as a renal mechanism for clearance of circulating cytokines. Based on these findings he is now approaching the mechanism of these phenomena utilizing the techniques of molecular biology. It is clear that certain mannose rich oligosaccharides regulated a number of important cellular activation signals including mRNA levels of c-FOS and IL-1. Dr. Muchmore's current data demonstrate that certain DNA binding proteins appear to be mannose specific lectins, and ultimately he believes that these oligosaccharides and potentially the cytokines IL-1, IL-2, and TNF are acting on transcriptional regulatory DNA binding proteins by either binding to or competing with defined mannose structures.

#### INSULIN-LIKE GROWTH FACTOR (IGF-I AND IGF-II) RECEPTORS

The insulin-like growth factors stimulate the growth of a variety of cells in culture. There is strong evidence from whole animal experiments that IGF-1 mediates many of the anabolic effects of pituitary growth hormone. The in vivo role for IGF-II is less clear, although high levels of IGF-II in fetal rat serum and the expression of IGF-II mRNA in many fetal tissues point to a role in fetal growth and development. The IGFs bind to two cell surface receptors. The IGF-I receptor binds IGF-I with higher affinity than IGF-II and also weakly recognizes insulin. The IGF-II receptor binds IGF-II with approximately 100-fold higher affinity than IGF-I and does not bind insulin even at very high concentrations. The two receptors have quite different structures as revealed initially by affinity labeling with radioligand and later by molecular cloning. The IGF-I receptor resembles the insulin receptor and consists of two alpha subunits which bind ligand and two beta subunits which traverse the membrane and possess tyrosine kinase domains on the cytoplasmic side. The IGF-II receptor is a large glycoprotein (300 kD) with 92% of the structure being extracellular and the small cytoplasmic portion having no homology with known tyrosine kinases. Molecular cloning, biochemical, and immunologic experiments have demonstrated that the IGF-II receptor is identical to the cation-independent mannose 6-phosphate receptor which targets over 50 lysosomal enzymes to lysosomes.

Experiments performed by Dr. Peter Nissley in collaboration with Dr. Gary Sahagian of Tufts University showed that the lysosomal enzyme,  $\beta$ -galactosidase



inhibited the binding of  $^{125}\text{I}$ -IGF-II to the IGF-II/Man-6-P receptor. However several other laboratories reported that mannose 6-phosphate itself actually increased the binding of  $^{125}\text{I}$ -IGF-II to the receptor. Dr. Nissley has performed additional experiments to explore the interaction of  $\beta$ -galactosidase and mannose 6-phosphate for binding of IGF-II to the receptor.  $\beta$ -galactosidase that was purified from bovine testis inhibited  $^{125}\text{I}$ -IGF-II binding to pure receptor with the concentration of  $\beta$ -galactosidase required for half-maximal inhibition being 25 nM. Inhibition of the enzymatic activity of  $\beta$ -galactosidase with D-galactonic acid gamma lactone did not affect the ability of  $\beta$ -galactosidase to inhibit the binding of  $^{125}\text{I}$ -IGF-II to the receptor. Acidic forms of  $\beta$ -galactosidase which exhibit higher cellular uptake via the IGF-II/Man-6-P receptor also exhibited maximal inhibition of binding of  $^{125}\text{I}$ -IGF-II to the receptor. The inhibition by  $\beta$ -galactosidase of IGF-II binding was reversed by mannose 6-phosphate and the dose response curve for reversal by mannose 6-phosphate was typical for binding of mannose 6-phosphate to the receptor. The results led to the conclusion that  $\beta$ -galactosidase inhibits the binding of IGF-II by binding to the mannose 6-phosphate recognition site on the receptor. Scatchard analysis of IGF-II binding in the presence of concentrations of  $\beta$ -galactosidase that partially inhibited IGF-II binding showed that  $\beta$ -galactosidase decreased the binding affinity for IGF-II, consistent with models in which  $\beta$ -galactosidase could change the conformation of the receptor or physically block the binding of IGF-II by binding to an adjacent site. Dr. Nissley found that the stimulation of  $^{125}\text{I}$ -IGF-II binding by mannose 6-phosphate was dependent on how the receptor had purified. Thus membranes, solubilized membranes, and receptors that had been purified on an IGF-II affinity column all exhibited the positive effect of mannose 6-phosphate on  $^{125}\text{I}$ -IGF-II binding, whereas receptor that had been purified on a lysosomal enzyme affinity column did not exhibit the mannose 6-phosphate effect. The ability of  $\beta$ -galactosidase to inhibit the binding of  $^{125}\text{I}$ -IGF-II was uniform for all of these preparations. The most direct explanation for these observations is that for the receptor preparations in which mannose 6-phosphate is stimulating  $^{125}\text{I}$ -IGF-II binding, mannose 6-phosphate is causing the dissociation of endogenous lysosomal enzymes from the receptors and thereby removing their inhibitory effect on IGF-II binding. Receptors purified on a lysosomal enzyme affinity column would have exchanged their endogenous lysosomal enzymes for the lysosomal enzyme attached to the matrix of the affinity column. In any case, stimulation of biologic effects by IGF-II acting through the IGF-II/Man-6-P receptor could be modulated by extracellular lysosomal enzymes or mannose 6-phosphate.

#### REGULATORY FUNCTION OF PROLINE AND ITS METABOLITE PYRROLINE-5-CARBOXYLATE

Dr. James Phang in previous studies showed that pyrroline-5-carboxylate (P5C) is a nutrient-dependent constituent of human plasma; 2) enters cells by its own uptake mechanism; and 3) has potent effector functions on cells including synergistic effects with PDGF. Therefore, he focused on production and release of P5C from cells and on the mechanisms by which P5C mediates signaling as a intercellular communicator.

P5C is released into the medium from a variety of cultured cells. Using a new assay for P5C based on its formation of an adduct with cysteine, he surveyed the medium conditioned by a variety of cell types including MCF-7, CHO, LLC-RK1, LLC-PK1 and several lines of normal human fibroblasts which all accumulated P5C in the medium with the exception of CHO which lack the enzymes for P5C synthesis. Human fibroblasts were the most active with P5C accumulating to levels of 2-3  $\mu\text{M}$  after 12 hours, levels which will produce some of the effector functions of P5C. The

accumulation of P5C was linear for at least 8 hours and plateaued after 24 hours. Glutamine was the main source of the P5C. In the absence of glutamine, P5C release fell to 10%. Although ornithine can also serve as the source of P5C, high concentrations are required (10  $\mu$ M). Furthermore, fibroblasts lacking ornithine aminotransferase, the enzyme converting ornithine to P5C showed comparable levels of P5C accumulation in the medium as normal fibroblasts. These studies show cultured cells can serve as a model for studying the release of P5C as an intercellular communicator.

To the previously characterized synergism between P5C and platelet-derived growth factor (PDGF) in stimulating the synthesis of PP-Rib-P can be added a marked stimulation of DNA synthesis. In the presence of 5% fetal bovine serum of 0.01 units of PDGF, P5C increased [<sup>3</sup>H]thymidine incorporation 50% over serum and 20% over PDGF. The effect was evident with [P5C] as low as 10  $\mu$ M and plateaued at 100  $\mu$ M. Interestingly, P5C could produce its effect when present for only the first hour of mitogenic activation suggesting that P5C could produce its effect when present for only the first hour of mitogenic activation suggesting that P5C was producing its effect on the signaling mechanism rather than by supplying ribonucleotides during S-phase.

More significant still, P5C markedly augmented the release of inositol phosphates. In cells prelabeled with [<sup>3</sup>H]myoinositol, labeled inositol phosphates released from membrane phospholipids can be quantitated by anion-exchange column chromatography. In quiescent human fibroblasts, fetal bovine serum rapidly increased the release of inositol phosphates. P5C, which has no effect by itself, markedly augmented the effect of serum. This stimulation was seen with concentrations as low as 5  $\mu$ M. Since P5C levels in human plasma as well as levels in conditioned tissue culture medium can attain levels of 2-3  $\mu$ M, these effects of P5C may be physiologically important.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04002-20 MET

## PERIOD COVERED

October 1, 1988 through September 30, 1989

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

~~Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunctions~~

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

PI:	Thomas A. Waldmann, M.D.	Branch Chief	MET, NCI
	Steven J. Greenberg, M.D.	Medical Staff Fellow	MET, NCI
	Claude Kasten-Sportes, M.D.	Medical Staff Fellow	MET, NCI
	Richard P. Junghans, M.D.	Medical Staff Fellow	MB, DCT, NCI
	Jack Burton, M.D.	Biotechnology Fellow	MET, NCI
	Angus Grant, Ph.D.	Biotechnology Fellow	MET, NCI
	Colin Glenn Begley, M.D.	Visiting Fellow	MET, NCI
	Craig Tendler, M.D.	Guest Researcher	MET, NCI
COOPERATING UNITS (if any)	Roger Garcia, M.D.	Guest Researcher	MET, NCI

Laboratory of Molecular Biology, NCI

Radiation Oncology Branch, NCI

LAB/BRANCH

Metabolism Branch

SECTION

## INSTITUTE AND LOCATION

DCRB, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

10.5

8.5

2

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects       (b) Human tissues       (c) Neither
- (a1) Minors      "B"      100%
- (a2) Interviews

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

Resting T cells do not express high-affinity IL-2 receptors, but receptors are rapidly expressed on T cells after activation with antigen or mitogen. We have identified two peptides that bind IL-2, the 55 kD peptide reactive with the anti-Tac monoclonal and a novel 70/75 kD (p75) peptide. We have proposed a multi-chain model for the high-affinity IL-2 receptor in which both the p55 Tac and the p75 IL-2 binding peptide are associated in the receptor complex. Using crosslinking, coprecipitation, and fluorescence energy transfer techniques, additional p22, p35, p40, p95, and p180 peptides have been identified that may be associated with the 55 kD Tac peptide in the multichain receptor. T cells in patients with certain lymphoid malignancies, select autoimmune disorders as well as individuals rejecting allografts express the IL-2 receptors identified by the anti-Tac monoclonal antibody whereas normal resting cells do not. To exploit this differential expression, a wide variety of IL-2 receptor-director therapeutic reagents have been generated including unmodified anti-Tac, toxin conjugates of anti-Tac, radioisotopic chelates of anti-Tac as well as hyperchimeric "humanized" anti-Tac antibodies that are entirely human except for the small hypervariable segments from the mouse antibody. The clinical application of these highly potent IL-2-receptor directed agents represents a new therapeutic perspective for certain neoplastic diseases, autoimmune disorders, and for the prevention of allograft rejection.



Project DescriptionMajor Findings:

Previously Dr. Thomas Waldmann and Dr. Stanley Korsmeyer introduced the analysis of immunoglobulin and T-cell receptor gene rearrangements to define the lineage (T or B cell) of cells lacking conventional markers, to establish whether abnormal lymphocytic populations are polyclonal, oligoclonal or monoclonal, and to broaden the scientific basis for the diagnosis and monitoring of the therapy of lymphoid neoplasia. More recently, Dr. Waldmann has exploited illegitimate chromosomal translocations in T cell leukemia involving the T cell receptor genes to clone elements involved in the regulation of normal and malignant cell proliferation. Dr. Glenn Begley, in collaboration with Dr. Ilan Kirsch, has identified a putative transforming gene which they call SCL-1 that is expressed in hematopoietic tissues. The gene was identified through the molecular cloning of a translocation between chromosomes 1 and 14;t(1:14)(p33;q11) that occurred in a human stem cell leukemia that was able to differentiate into myeloid and lymphoid lineages. As a consequence of the translocation, a novel fusion transcript was generated between the sequences on chromosome 1 and chromosome 14. The gene of chromosome 1 manifested a DNA binding motif shared with the *Drosophila* daughterless gene and the myc and MyoD proteins. This gene was expressed in fetal liver and normal bone marrow and may be important for hematopoietic stem cell development and oncogenesis.

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One of Dr. Waldmann's most critical contributions was his recognition that the IL-2 receptor represents an extraordinarily versatile therapeutic target. He entered this area to exploit his observations that T cells in patients with certain lymphoid malignancies (e.g., adult T cell leukemia, cutaneous T cell lymphoma, hairy cell leukemia and Hodgkin's disease) and select autoimmune diseases (e.g., scleroderma, tropical spastic paraparesis and rheumatoid arthritis) as well as individuals rejecting allografts express the IL-2 receptors identified by the anti-Tac monoclonal antibody whereas normal resting cells and their precursors do not. He reasoned that agents that could eliminate Tac-expressing leukemic cells or activated T cells involved in other disease states could be effective therapies for these disorders yet would retain the Tac-negative mature normal T cells and their precursors. Dr. Waldmann and his coworkers have developed a wide variety of therapeutic reagents. Dr. Waldmann has effectively utilized the unmodified anti-Tac monoclonal antibody to block binding of IL-2 to its growth factor receptor and to lead to remissions lasting 1, 5 and over 8 months in the treatment of



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Honors and Awards:

- 1988 Morton Grove-Rassmussen Lecture to the International Symposium on Immunoglobulins (Amsterdam)  
 1988 Rutgers Biology Colloquium Lecture  
 1989 Elected to Rank of Fellow, American Association for the Advancement of Science  
 1989 Elected to the American Academy of Arts and Sciences

Publications:

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04004-27 MET

## PERIOD COVERED

October 1, 1988 through September 30, 1989

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

Amino Acids and Growth Factors in Cell Activation

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

PI:	James M. Phang	Senior Investigator	MET, NCI
	A. James Mixson	Medical Staff Fellow	MET, NCI

## COOPERATING UNITS (if any)

Grace C. Yeh, CPB, DCT, NCI

David Valle, M.D., Johns Hopkins Hospital School of Medicine, Baltimore, MD

LAB/BRANCH

Metabolism Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH Bethesda, MD

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

5

2

3

## CHECK APPROPRIATE BOX(ES)

<input checked="" type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	
<input checked="" type="checkbox"/> (a1) Minors			"B" 100%
<input type="checkbox"/> (a2) Interviews			

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

The role of pyrroline-5-carboxylate (P5C) in nutrition-dependent intercellular communication and transmembrane signaling has been previously demonstrated. Our current studies have extended our previous findings and have elucidated the following mechanisms. 1) P5C was released into the culture medium by a number of cell lines. Glutamine and ornithine both could be precursors for the formation and release of extracellular P5C but glutamine was the major source. Concentrations in conditioned medium reached 2-3  $\mu\text{M}$ , a level which can mediate a number of effector functions. 2) We have identified a 50 kilodalton membrane protein which may be the specific membrane carrier protein for P5C. Previous work showed that the entry of P5C into cells is mediated by its own special carrier mechanism which transfers oxidizing potential *pari passu* with P5C entry. Using labeled p-chloromercuri-benzosulfonate, we have identified a band on SDS PAGE which shows the same sensitivity to specific inhibitors as does P5C uptake. 3) P5C stimulated thymidine incorporation in cells activated by growth factors. We previously showed that PP-Rib-P production is markedly stimulated by P5C and that PDGF interacts synergistically with P5C. This synergism also applies to the incorporation of [ $^3\text{H}$ ]thymidine. 4) The mechanism of these P5C effector functions involved the synergistic stimulation of phosphoinositides. P5C markedly increases the production of inositol phosphates over that produced by growth factors. These studies further demonstrated that P5C serves as a mechanism for integrating nutritional influences with growth factor-stimulated cellular events.

Project DescriptionMajor Findings:

1. The release of pyrroline 5-carboxylate from cultured cells into medium.
  - a. A new method for measuring pyrroline 5-carboxylate (P5C) in conditioned media.

Our ongoing studies on P5C as an intercellular communicator will be greatly aided by the development of a new method for measuring P5C. This method takes advantage of the interaction between P5C and cysteine forming an adduct which can be isolated by cation-exchange column chromatography. Although the exact nature of the adduct remains undefined, evidence suggests that it is a thioester. With [<sup>35</sup>S]cysteine the recovery of label in adduct fractions is quantitatively dependent on the amount of P5C. The formation of the adduct is rapid, plateaus by 6 minutes and is optimal at pH 6.8. Specificity of formation of the adduct was shown by column exchange chromatography of samples and assaying each fraction. Adduct formation co-migrated with radioactive P5C. No other fractions contained measurable adduct. A quantitative comparison of this new assay with a published P5C reductase-catalyzed assay showed values which were virtually identical. Although this new assay is less sensitive than the P5C reductase-catalyzed assay, it is more efficient and requires only commercially available reagents.

- b. P5C release by cultured cells.

Previous work from our laboratory showed that P5C circulates in human plasma as a nutrient-dependent constituent. We studied the synthesis and release of P5C by cells into culture medium to elucidate the regulation of this regulatory molecule. A variety of cells, MCF-7, LLC-RK1, LLC-PK1 and several lines of normal human fibroblasts, accumulate P5C in the medium. The concentration of P5C increases linearly with time for 8 hours after which it increases curvilinearly. The highest levels, 2-3  $\mu\text{M}$ , were seen with fibroblasts. Glutamine was the main source of the P5C. In its absence, P5C accumulation fell to 10%. Although ornithine can also serve as the source of P5C, high concentrations are required (10  $\mu\text{M}$ ). Furthermore, fibroblasts lacking ornithine aminotransferase, the enzyme converting ornithine to P5C, showed comparable levels of P5C accumulation as normal fibroblasts. These levels in medium are approaching the concentrations of P5C which produce stimulatory effects on cells, a finding which suggests that the efflux and influx of P5C may play a regulatory role in cell physiology.

2. The effector functions of P5C.
  - a. P5C stimulation of thymidine incorporation.

We previously characterized the synergistic stimulation of phosphoribosyl pyrophosphate (PP-Rib-P) by platelet-derived growth factor (PDGF) and P5C. Our current work shows that P5C also stimulates the incorporation of thymidine. In the presence of 5% fetal bovine serum or .01 units/ml of PDGF to activate quiescent fibroblasts, P5C increased [<sup>3</sup>H]thymidine incorporation 50% over FBS and 20% over PDGF but was without effect by itself. The effect was evident with [P5C] as low as 10  $\mu\text{M}$  and plateaued at 100  $\mu\text{M}$ . The half-maximal concentration was 15-50  $\mu\text{M}$ .

Critical factors in eliciting this effect are: 1) The presence of P5C must be restricted to early G1. In late G1, P5C can be inhibitory. Importantly, stimulation occurred with as little as 30 minutes of exposure. 2) P5C stimulated only in subconfluent cells ( $5 \times 10^3$  cells/cm<sup>2</sup>) in which the entry into S is somewhat later than in confluent cells. 3) In confluent cells, P5C can be inhibitory. These findings implicate an interaction of P5C with signaling mechanisms rather than with the synthesis of ribonucleotides.

b. The effect of P5C on inositol phosphates.

Previous work on the synergism between P5C and PDGF in stimulating PP-Rib-P suggested an interaction at the level of phosphoinositide turnover. Our recent studies showed that P5C markedly augmented the release of inositol phosphates. In cells prelabeled with [<sup>3</sup>H]myoinositol, labeled inositol phosphates released from membrane phospholipids were quantitated by anion-exchange column chromatography. P5C, by itself, was without effect, but it increased inositol phosphates by 50% over that of 5% FBS alone. The stimulation was seen with [P5C] as low as 5  $\mu$ M and plateaued at 50  $\mu$ M. Half-maximal concentration was about 10  $\mu$ M. Since P5C levels in human plasma as well as in conditioned medium can attain levels of 2-3  $\mu$ M, these effects of P5C on cultured cells may be physiologically important.

c. Identification of the P5C membrane carrier protein.

Previous studies showed that P5C enters through its own membrane carrier which does not recognize any of the naturally-occurring amino acids. However, it does recognize certain structural analogues to P5C, e.g., 2-pyridine carboxaldehyde (2PC). Furthermore, P5C uptake is sensitive to inhibition by low ( $\mu$ M) concentrations of p-chloromercuribenzenesulfonate (PCMBs) which inactivates cell surface proteins by reacting with free sulfhydryl groups. The presence of P5C or P2C, presumably by binding to the P5C carrier, protects against this effect of PCMBs. Using [<sup>203</sup>Hg]PCMBs incubated with cells in the presence and absence of P5C or P2C, we were able to identify a 50 kilodalton band which was sensitive to P5C and P2C.

Publications:

Mixson AJ, Phang JM. The uptake of pyrroline 5-carboxylate: group translocation mediating the transfer of reducing-oxidizing potential. *J Biol Chem* 1988;263:10720-4.

Yeh GC, Phang JM. Stimulation of phosphoribosyl pyrophosphate and purine nucleotide production by pyrroline 5-carboxylate in human erythrocytes. *J Biol Chem* 1988;263:13083-89.

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Merrill M, Yeh GC, Phang JM. Purification and characterization of human erythrocytes pyrroline-5-carboxylate reductase. *J Biol Chem* 1989, in press.

Flynn MP, Martin MC, Moore PT, Stafford JA, Fleming GA, Phang JM. Type II hyperprolinaemia in a pedigree of Irish travels (nomads). *Arch Dis Childhood* London, 1989, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04015-18 MET

## PERIOD COVERED

October 1, 1988 through September 30, 1989

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

Development and Function of Humoral and Cellular Immune Mechanism

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

PI:	R. Michael Blaese, M.D.	Section Chief	MET, NCI
	Kenneth Culver, M.D.	Medical Staff Fellow	MET, NCI
	W. French Anderson, M.D.	Chief	LMH, NHLBI
	Steven A. Rosenberg, M.D., Ph.D.	Chief	SB, NCI
	Richard Clark, M.D.	Chief	CSB, NHLBI
	Christopher Stone, M.D.	Medical Staff Fellow	LMH, NHLBI
	George Abraham, M.D.	Guest Researcher	NIAID, NCI

## COOPERATING UNITS (if any)

LMH, NHLBI	NIAID
CSB, NHLBI	SB, NCI

LABORATORY  
Metabolicism BranchSECTION  
Cellular Immunology SectionINSTITUTE AND LOCATION  
Bethesda, Maryland

TOTAL MAN-YEARS:	4	PROFESSIONAL:	2	OTHER:	2
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## CHECK APPROPRIATE BOX(ES)

(a) Human subjects     
  (b) Human tissues     
  (c) Neither "B" 100%  
 (a1) Minors  
 (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The development of techniques to facilitate gene therapy in man has continued to be a major emphasis of our work. Using a retroviral gene transfer system, we have successfully transferred the gene for adenosine deaminase into ADA deficient T cells *in vitro* and totally corrected the biochemical defect in these cells. Using a bone marrow transplantation model in monkeys, we have reconstituted irradiated monkeys with autologous marrow which has been treated with the human ADA retrovirus vector. These monkeys were found to express human ADA in their peripheral blood cells for up to 120 days after reconstitution. The level and duration of expression are insufficient to attempt human reconstitution at this time but the results suggest that this approach may ultimately provide a successful mechanism for the treatment of a variety of genetic disorders of man. We have also used cultured lymphocytes and fibroblasts as cellular vehicles for gene transfer in animals. With T cells as the carriers of transduced genes, mice have continued to express human ADA, rat growth hormone, and neomycin phosphotransferase for many weeks. Dr. Blaese and colleagues Steven A. Rosenberg and W. French Anderson have recently performed the first authorized gene transfer experiment in man using the neoR gene to mark T lymphocytes used in cancer therapy. Our studies developing and defining the new immunosuppressive drug succinylacetone (SA) have continued. We have found that this agent is perhaps the most potent T and B cell immunosuppressive compound available, blocking tumor allograft rejection and preventing GVHD during total allogeneic bone marrow transplantation while allowing normal engraftment and reconstitution to occur. SA is a very potent inhibitor of both primary and secondary antibody responses. Trials of SA in a rat model of autoimmune eye disease have shown that it is remarkably effective in preventing a type of uveitis that usually results in blindness in experimental animals. SA has been shown to prevent rejection of cardiac allografts in rats and preliminary experiments in primates have shown it to be effective in preventing antibody production and cardiac allograft rejection.

Project DescriptionMajor Findings:

A major effort of the Cellular Immunology Section has continued to focus on the development of techniques of gene transfer which will be useful for the application of gene therapy in man. Several systems have been studied including retroviral mediated gene transfer into bone marrow progenitor cells, cultured fibroblasts, transformed T cells and primary antigen specific IL-2 independent T cell lines from mice, rats, monkeys and IL-2 dependent T cells from man. We have been successful in transferring the gene for adenosine deaminase into ADA deficient T cell lines and totally correcting the biochemical defect in these cells in vitro. Using autologous bone marrow transplantation in lethally irradiated monkeys, we have been successful in transferring the gene for human ADA into these monkeys. Expression of the human enzyme in the peripheral blood mononuclear cells of the recipient animals was detected within 45 days of transplantation and persisted for another 100 days. These results are very encouraging, but the level of expression of human enzyme was low and not sustained for a long enough period to permit application of present techniques to the therapy of human disease. To avoid some of the problems encountered with bone marrow gene therapy, we have been developing the alternative approach of using lymphocytes and fibroblasts as cellular vehicles for gene transfer. Lymphocytes have several potential advantages for gene therapy. Cultured lymphocytes are readily infected with retroviral vectors, can be selected for appropriate gene expression in culture, and can be thoroughly tested before reintroduction into the body. The antigen specificity of lymphocytes can also be utilized to expand the population of gene transduced cells in vivo by simple immunization or to target the introduced gene to certain specific sites in the body such as directing antineoplastic factors to the sites of tumor metastases. We have shown that murine antigen specific T cells transduced with the genes for neomycin resistance and human ADA will survive for many weeks after reintroduction into intact animals and will continue to express both genes at high levels. Lymphocytes and fibroblasts transduced with the gene for growth hormone have been reintroduced into hypophysectomized mice and rats and result in the production of growth hormone in these animals. Gene therapy combined with autologous bone marrow transplantation will be important for the treatment of many inherited human diseases, but it may well be that cultured lymphocytes and/or fibroblasts will be the first cellular vehicles bringing clinical application of gene transfer as a therapeutic procedure.

We have continued with our long-time interest in the Wiskott-Aldrich syndrome with studies of platelet function before and after splenectomy, changes in platelet cell membrane glycoproteins occurring after splenectomy, detailed lymphocyte phenotype analysis of both T cells and B cells, family genetic linkage analysis using X-chromosome RFLPs, and carrier detection based on the pattern of X-inactivation found in peripheral cells of the carriers. This latter study is based on the observation that the affected boys have defects in T cell, B cell, monocyte, granulocyte, and platelet function and yet the carrier females have no detectable defects in any of these lineages. Since all females are mosaics for traits inherited on the X-chromosome because of random inactivation of one of her two X-chromosomes (lyonization) occurring in the early embryo, carriers might be expected to demonstrate abnormalities unless all their cells were derived from precursors in which the chromosome carrying the defective gene was inactivated. We have studied

this question using RFLPs for the probes to the X-linked genes PGK and HPRT to distinguish the maternal from the paternally inherited X-chromosome and methylation sensitive restriction endonucleases to distinguish the active from the inactive X-chromosomes. Whereas approximately half the cells from normal females express the genes on the paternally derived X and the remaining express genes from the maternally derived X-chromosome, the T cells, B cells, and granulocytes from obligate carriers of the WAS gene were found to be using only 1 of the 2 chromosomes in all their peripheral cells. This strikingly unbalanced pattern of X-inactivation was not seen in any of the more than 80 normal females studied and thus appears to represent a reliable marker for the carrier state in these females.

Our studies of the immunosuppressive compound succinylacetone have continued to provide exciting information about this most potent of immunosuppressive agents. Succinylacetone is a seven carbon organic acid. It is an inhibitor of the second step of heme biosynthesis and has been shown to have inhibitory effects on the growth of certain tumors *in vitro*. We initially demonstrated that the compound appeared to have potent immunosuppressive activity in the rat because it was capable of preventing the rejection of tumor allografts. More recent studies showed that succinylacetone behaves as a profound suppressive agent for T cell mediated immune functions. It not only blocks tumor and other forms of allograft rejection but also prevents graft vs. host disease induced in adult Fl animals after the injection of parental strain lymphoid cells. In a model of bone marrow transplantation in fully allogeneic adult rats, succinylacetone treatment not only prevented lethal graft vs. host disease in the transplanted animals but also permitted normal engraftment and full reconstitution. The striking finding is that the treatment required to prevent graft vs. host disease was limited to 24 days of duration and yet the animals remained fully chimeric for at least 300 days post bone marrow transplantation. Immune competence returned to the transplanted animals in a timely fashion, and survival in the recipients of allogeneic cells was equivalent to the survival in transplant recipients receiving syngeneic bone marrow. Succinylacetone has been shown to have profound effects on the B lymphocyte system as well. Initially, it was shown to inhibit the production of antibodies to sheep erythrocytes in rats treated for a period of 2 weeks. We have extended these initial studies to demonstrate that the drug has immunosuppressive effects in mice as well. Using a series of model antigens that are both helper T cell dependent and helper T cell independent type I and type II antigens, we have shown that succinylacetone interferes with the production of antibodies to all classes of antigen. Therefore its inhibitory effects cannot be explained solely by the inhibition of T cell function. Thus, succinylacetone is one of the very few agents that has profound effects on the production of antibodies and immunoglobulin. In a series of collaborative studies with Alan Palestine of the National Eye Institute, we have been treating Lewis rats that have been injected with purified retinal protein that induces a model disorder of inflammatory uveitis. 100% of Lewis rats develop blindness within 14 days of treatment with Freund's containing purified retinal protein. Antibodies can be detected to the retinal protein within 7 days, and usually by 10 days after immunization, inflammatory changes can be found involving the retina as well as other optical structures. Animals treated with succinylacetone from the first day of retinal protein injection were totally protected from the development of inflammatory uveitis. Even if the inflammatory uveitis process were allowed to initially develop to the point that antibodies to the retinal protein had been produced, initiation of succinylacetone therapy at 7-8 days was still able to totally prevent



the destructive retinitis associated with this animal model. This form of therapy is the most effective in this animal model, exceeding the effectiveness of cyclosporin A, the current agent of choice in the therapy of autoimmune disorders. The mechanism of action of succinylacetone is unclear. Although the agent has an antiproliferative effect to T cells and B cells in vitro, the dose required in vitro also inhibited the proliferation of non-lymphoid cells, such as fibroblasts and tumor cells. The observation that bone marrow engraftment proceeds normally in the presence of profoundly immunosuppressive effects also suggest that this is not a general antiproliferative type agent. Preliminary studies of adding various growth factors to cells in vivo and in vitro have been unable to overcome the immunosuppressive effects of the drug. We have extensively analyzed histology and blood chemistries in animals treated with toxic doses of succinylacetone and have found no consistent pathological finding other than a profound depletion of the lymphoid areas in all tissues. With 14 days of high dose succinylacetone therapy, the thymus of treated animals almost totally involutes, giving the histological appearance of the thymus seen in infants with one of the forms of SCID. Thus succinylacetone in addition to being a profound and potentially useful clinical immunosuppressive drug, may provide us with a mechanism for studying a variety of cellular pathways.

#### Publications:

Kohn DB, Anderson W, Blaese RM. Gene therapy for genetic diseases. Cancer Investigation, in press.

Blaese RM, Lane EC. AIDS and other immunodeficiency diseases. In: Kelley WN, Harris ED, Ruddy S, Sledge CD, eds. Textbook of rheumatology, 3rd Ed. Philadelphia: WB Saunders, 1989;1355-85.

Blaese RM, Poplack DP, Muchmore AV. Disorders of the mononuclear phagocyte system. In: Stiehm R, Fulgeniti V, eds. Immunologic disorders of infants and children, 3rd Ed, Chapter 16. Philadelphia: WB Saunders, 1989;364-83.

Douglas S, Hassan NF, Blaese RM. The mononuclear phagocytes. In: Stiehm R, Fulgeniti V, eds. Immunologic disorders of infants and children, 3rd Ed, Chapter 6. Philadelphia: WB Saunders, 1989;81-96.

Blaese RM. Genetic immunodeficiency syndromes with defects in both T and B lymphocyte functions. In: Scriver DR, Beaudet AL, Sly WS, Valle D, eds. The metabolic basis of inherited disease, 6th Ed. New York: McGraw-Hill, 1989;2697-2709.

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Kohn DB, Mitsuya H, Ballow M, Selegue JE, Barankiewicz J, Cohen A, Gelfand E, Anderson WF, Blaese RM. Establishment and characterization of adenosine deaminase (ADA) deficient human T cell lines. J Immunol 1989;142:3971-77.

Eglitis MA, Kantoff PW, Kohn DB, Karson E, Moen RM, Lothrop CD Jr, Blaese RM, Anderson WF. Retroviral-mediated gene transfer into hematopoietic cells. Tavassoli M, ed. 3rd Symposium on molecular biology of hemopoiesis. New York: Plenum. (In press)

Carpenter NJ, Filipovich A, Blaese RM, Carey TL, Berkel I. Variable immunodeficiency with abnormal condensation of the heterochromatin of chromosomes 1, 9, and 16. *J Pediat* 1988;112:757-60.

Blaese RM, Hong R. Combined immunodeficiency syndromes. In: Oski FA, DeAngelis C, Feigin P, Warshaw JB, eds. Principles and practice of pediatrics. Philadelphia: Lippincott. (In press)

Kwan S-P, Sandkuyl LA, Blaese M, Kunkel L, Bruns G, Parmley R, Skarshoug S, Page DC, Ott J, Rosen FS. Genetic mapping of the Wiskott-Aldrich syndrome with two highly-linked polymorphic DNA markers. *Genomics* 1988;3:39-43.

Straus SE, Dale JK, Tobi M, Lawley T, Preble O, Blaese RM, Hallahan C, Henle W. Acyclovir treatment of the chronic fatigue syndrome: lack of efficiency in a placebo-controlled trial. *N Engl J Med* 1988;319:1692-98.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04016-16 MET

## PERIOD COVERED

October 1, 1988 through September 30, 1989

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, NGI
Mark Sklar	Medical Staff Fellow	MET, NCI
Wlodzimierz Lopaczynski	Fogarty Visiting Fellow	MET, NCI
Barbara Linder	Medical Staff Fellow	NIH/D

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolism Branch

## SECTION

Endocrinology Section

## INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

3

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

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

"B" 100%

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

We have investigated the interaction of the two classes of ligands (lysosomal enzymes and IGF-II) which bind to the IGF-II/Man-6-P receptor. Highly purified  $\beta$ -galactosidase which we isolated from bovine testis inhibited  $^{125}\text{I}$ -IGF-II binding to pure IGF-II/Man-6-P receptor with the concentration that was required for half-maximal inhibition being 25 nM. The inhibition by  $\beta$ -galactosidase was reversed by mannose-6-phosphate and the dose response curve for the reversal by mannose-6-phosphate was typical for binding to the IGF-II/Man-6-P receptor. Acidic forms of  $\beta$ -galactosidase were isolated by ion exchange chromatography on DEAE-Sephacel. These acidic forms of  $\beta$ -galactosidase which had been previously shown to exhibit enhanced cellular uptake also showed increased potency in inhibiting the binding of  $^{125}\text{I}$ -IGF-II to the receptor. Scatchard analysis of binding of IGF-II to the receptor in the presence of concentrations of  $\beta$ -galactosidase which caused partial inhibition of  $^{125}\text{I}$ -IGF-II binding, demonstrated that  $\beta$ -galactosidase decreased the binding affinity for IGF-II. We have shown that IGF-II is a substrate for Cathepsin C. Cathepsin C cleaves Ala-Tyr depeptide from the N-terminus. Comparison of the native IGF-II with the desdipeptidyl IGF-II for binding to the IGF-II/Man-6-P receptor, the IGF-I receptor, and IGF binding proteins showed equipotency of the two species of IGF-II. We have also attempted to confirm a report that in the 1854 rat cell line which produces IGF-II and has IGF-II receptors, IGF-II is an autocrine growth factor for of these cells in serum free media, acting through the IGF-II/Man-6-P receptor. We found that neither IGF-I or IGF-II could support the multiplication of the 1854 cell line under serum-free conditions and, using an antibody which blocks the binding of IGF-II to the IGF-II/Man-6-P receptor, we were unable to demonstrate a role for this receptor in signaling the growth response.

Project DescriptionMajor Findings:

Evidence that the lysosomal enzyme,  $\beta$ -galactosidase, inhibits the binding of  $^{125}\text{I}$ -IGF-II to the IGF-II/Man-6-P receptor by binding to the mannose 6-phosphate recognition site.

During a collaborative study with Gary Sahagian, Tufts University, on the biochemical and immunologic comparison of the IGF-II receptor and the mannose 6-phosphate receptor, it was demonstrated that the lysosomal enzyme,  $\beta$ -galactosidase, inhibited the binding of  $^{125}\text{I}$ -IGF-II to the mannose 6-phosphate receptor as measured by affinity crosslinking. We have performed additional experiments to further characterize the inhibition of IGF-II binding by  $\beta$ -galactosidase. We demonstrated that incubation of  $\beta$ -galactosidase with  $^{125}\text{I}$ -IGF-II did not result in degradation of the radioligand as measured by trichloroacetic acid precipitation. In addition, inhibition of the enzymatic activity of  $\beta$ -galactosidase with D-galactonic acid gamma lactone did not affect the ability of  $\beta$ -galactosidase to inhibit the binding of  $^{125}\text{I}$ -IGF-II to the receptor. Thus the ability of  $\beta$ -galactosidase to inhibit the binding of IGF-II to the receptor is not attributable to contaminating proteases or the potential alteration of the carbohydrate portion of the receptor by  $\beta$ -galactosidase acting as an enzyme. We purified  $\beta$ -galactosidase from bovine testis by affinity chromatography, first on Concanavalin A-Sepharose and then on p-aminobenzyl-1-thio- $\beta$ -D-galactopyranoside-Agarose. We then performed ion exchange chromatography on DEAE-Sephacel to isolate acidic forms of the enzyme which had been shown to exhibit high cellular uptake via the IGF-II/Man-6-P receptor. There was a 2.6-8 fold enhancement in the ability of the high uptake forms of the enzyme to inhibit the binding of  $^{125}\text{I}$ -IGF-II to the receptor. Preparations of high uptake forms of  $\beta$ -galactosidase inhibited the binding of  $^{125}\text{I}$ -IGF-II to preparations of pure receptor with the concentration required for half-maximal inhibition of binding being 25 nM. Co-incubation with mannose 6-phosphate prevented the inhibition of  $^{125}\text{I}$ -IGF-II binding by  $\beta$ -galactosidase and the dose response curve for reversal by mannose 6-phosphate was characteristic for interaction of mannose 6-phosphate with the IGF-II/mannose 6-phosphate receptor. Scatchard analysis of IGF-II binding to pure receptor in the presence of concentrations of  $\beta$ -galactosidase which caused partial inhibition of  $^{125}\text{I}$ -IGF-II binding, demonstrated that  $\beta$ -galactosidase caused a decrease in binding affinity for IGF-II. This result would be consistent with models in which  $\beta$ -galactosidase inhibits binding of IGF-II by binding to an adjacent site or in which  $\beta$ -galactosidase binds at a distal site and changes the conformation of the receptor. In any case, it seems clear that  $\beta$ -galactosidase inhibits the binding of IGF-II to the receptor by binding to the mannose 6-phosphate recognition site. Thus lysosomal enzymes could modulate both the cellular uptake of IGF-II via the IGF-II/Man-6-P receptor and signaling by IGF-II through this receptor.

IGF-II is a substrate for Cathepsin C

While testing whether or not the inhibition of  $^{125}\text{I}$ -IGF-II binding to the IGF-II/Man-6-P receptor by various lysosomal enzymes could be explained by digestion of the radioligand by the hydrolases, we observed that incubation of rat  $^{125}\text{I}$ -IGF-II with Cathepsin C resulted in the conversion of a fraction of the radioligand to a



trichloroacetic acid soluble form. Analysis of the digestion products by reversed phase high pressure liquid chromatography, revealed the presence of a species which eluted slightly before the untreated radioligand. Rat IGF-II isolated from the media conditioned by a rat liver cell line BRL 3A had been shown by others to be an approximately 50:50 mixture of two species having amino termini of Ala-Tyr-Arg-Pro-Ser.... or Tyr-Arg-Pro-Ser.... Based on the properties of Cathepsin C it would be predicted that the IGF-II species with an N terminal Ala would be a substrate, the enzyme cleaving off the dipeptide Ala-Tyr and then stopping at Arg. If the tyrosine residue in the cleaved dipeptide had been radiolabeled with  $^{125}\text{I}$ , then this would explain the increase in trichloroacetic acid solubility of the radiolabeled IGF-II following treatment with Cathepsin C. The IGF-II species beginning with Tyr-Arg-Pro-Ser... would not be expected to be a substrate for Cathepsin C because of the proline residue in the third position. To test this prediction, unlabeled rat IGF-II was treated with Cathepsin C and the species with the shorter elution time on reversed phase HPLC was isolated. N terminal amino acid sequencing of this species performed by William Burgess, the Jerome Holland Red Cross Laboratory, confirmed that the species began with Arg-Pro-Ser as predicted. In addition, human IGF-II which has an amino terminal sequence of Ala-Tyr-Arg-Pro-Ser... was treated with Cathepsin C. All of the IGF-II was converted to a species with a mobility on reversed phase HPLC which was slightly faster than the untreated IGF-II. The amino terminal sequence of this species was Arg-Pro-Ser as predicted. We compared the native human IGF-II and the desdipeptidyl IGF-II in a variety of assays that assessed the ability of these two species to interact with IGF receptors and IGF binding proteins. The two species were indistinguishable in binding to the IGF-II/Man-6-P receptor from rat placenta, the IGF-I receptor from human placenta, the 150 kDa binding protein from adult rat serum, and the 40 kDa binding protein from 3 day postnatal rat serum. In addition, two IGF-II species were equipotent in stimulating  $^3\text{H}$  thymidine incorporation into DNA in MG63 cells, a human osteosarcoma cell line in which stimulation of DNA synthesis by IGFs is signaled through the IGF-I receptor. Thus removal of the N terminal two amino acids from IGF-II does not result in loss of ability to bind to IGF binding proteins or IGF receptors. This is of interest in light of the report that desdipeptidyl IGF-I has greatly reduced binding affinity for one of the IGF binding proteins.

#### IGFs and the growth of the rat cell line 1854 in serum-free medium

Rosenfeld and his colleagues reported that the rat cell line 1854 which produces IGF-II, has IGF-II/Man-6-P receptors and responds to IGF-II with an increase in cell number. Furthermore these workers reported that an antiserum directed against IGF-II/Man-6-P receptor blocked the multiplication of 1854 cells in serum-free medium. Because of their conclusion that the IGF-II/Man-6-P receptor was signaling cell multiplication in this cell line, we attempted to confirm these observations. We found that if the serum-free medium was changed every 24 hours the cells multiplied just as rapidly as when the medium was left unchanged over a several day period. However, if the medium was changed every 12 hours, total cell number did not increase. In the cultures in which the medium was changed every 12 hours, cells died around the periphery of the culture, while cells in the center continued to multiply. Thus the failure to increase cell number represented the summation of cell multiplication and cell death. When IGF-II or IGF-I was added to the cells in the media change every 12 hours, there was no increase in cell number; instead, cell number actually declined after several days in culture in the presence of

IGF-I or IGF-II. The rate of cell multiplication in the center of the cultures as measured by autoradiography following  $^3\text{H}$ -thymidine incorporation was not enhanced by addition of IGF-I or IGF-II. Furthermore the rate of cell multiplication as measured by cell number under conditions where the cells were growing maximally (media change every 24 hours) was not decreased by addition of an anti-IGF-II/Man-6-P receptor IgG at concentrations which blocked binding of  $^{125}\text{I}$ -IGF-II to the IGF-II/Man-6-P receptor. We conclude that IGF-II does not appear to be an autocrine growth factor or survival factor for these cells although IGF-II is being produced.

Publications:

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Kiess W, Thomas GL, Greenstein LA, Lee L, Sklar MM, Rechler MM, Sahagian GG, Nissley SP. Insulin-like growth factor-II (IGF-II) inhibits both the cellular uptake of  $\beta$ -galactosidase and the binding of  $\beta$ -galactosidase to purified IGF-II/mannose 6-phosphate receptor. *J Biol Chem* 1989; 4710-14.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04017-11 MET

## PERIOD COVERED

October 1, 1988 through September 30, 1989

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

Biology of the Immune Response

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

PI: David L. Nelson Head, Immunophysiology Section

MET, NCI

William D. Schwieterman Medical Staff Fellow

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolism Branch

## SECTION

Immunophysiology

## INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

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

"B" 100%

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

Studies were performed to examine the maturation and regulation of the human immune response in normal individuals and in patients with congenital and acquired immune deficiency states associated with a high frequency of cancer. The interaction of the T-cell derived lymphokine interleukin-2 (IL-2) with its cell membrane receptor (IL-2R) plays a pivotal role in the establishment and maturation of the immune response. Elevated levels of soluble Tac protein were found in the serum of several retroviral associated diseases including the adult T-cell leukemia (ATL), hairy cell leukemia (HCL), and the acquired immune deficiency syndrome (AIDS). Favorable responses to therapy in ATL and HCL were associated with reductions in serum Tac protein. Measurement of Tac protein in serum is thus useful in the diagnosis and management of certain cancer patients and in monitoring the state of immunologic activation in humans *in vivo*. In a continuing effort to define the nature of certain immunodeficiency disorders and to gain insight into human B-lymphocyte maturation, we have initiated molecular genetic studies of a kindred with X-linked hypogammaglobulinemia and isolated growth hormone deficiency. Mapping studies by restriction fragment length polymorphism have shown that this disease is due to a gene distinct from that causing the more common form of X-linked agammaglobulinemia or Bruton's disease. Another unique human immunodeficiency associated with a prevalence of T-cells expressing the gamma and delta chains of the human T-cell receptor for antigen was identified and characterized. The study of immunodeficiency disorders provides unique molecular insights into the maturation of B-cells and T-cells in man.



Project DescriptionMajor Findings:

The cell membrane receptor for the T-cell derived lymphokine, interleukin-2 (IL-2) is a multichain structure consisting of at least two proteins with molecular weights of 55 and 75 kilodaltons (kDa). Hybridoma-derived monoclonal antibodies binding to distinct epitopes on the 55 kDa molecule have been produced and this chain has been termed the Tac protein. The soluble Tac protein is 10 kDa smaller in size than the cell-membrane form of this molecule and specifically binds IL-2, albeit at low affinity (20 nM).

Using two monoclonal antibodies directed against distinct epitopes on the Tac protein, we constructed a "sandwich" enzyme-linked immunoassay (ELISA) to quantitatively measure soluble Tac molecules. Soluble Tac protein was measurable in the serum and urine but not cerebrospinal fluid of all normal individuals. Since soluble Tac protein was produced by activated normal lymphocytes and monocytes as well as constitutively produced by certain tumor cell lines, we undertook studies to measure the level of Tac protein in body fluids of certain patients with cancer and other patients whose immune system might be undergoing activation in vivo.

Elevated levels of soluble Tac protein were observed in diseases associated with human retroviral infections including the adult T-cell leukemia (ATL), hairy cell leukemia (HCL), and the acquired immune deficiency syndrome (AIDS). Elevations of soluble Tac protein were also observed in several groups of patients with neurological diseases including multiple sclerosis and schizoprenia. The measurement of Tac protein from the human IL-2 receptor in various body fluids is useful in the diagnosis and management of patients with neoplastic and inflammatory disorders.

While the genes encoding foreign antigen binding immunoglobulin molecules on B-cells (Ig) have been molecularly cloned, our knowledge of the regulation and maturation of the expression of these genes is still very limited. One example of abnormal expression of these genes occurs in the primary immunodeficiency diseases such as X-linked hypogammaglobulinemia and isolated growth hormone deficiency where B-cell maturation is apparently blocked at the pre-B-cell level. Therefore a molecular understanding of the cause of disordered gene expression in these patients might provide important information as to the regulation of the expression of immunoglobulin genes during normal development. Mapping studies by restriction fragment length polymorphism have shown that X-linked hypogammaglobulinemia and isolated growth hormone deficiency is due to a gene distinct from that causing the more common form of X-linked agammaglobulinemia or Bruton's disease. Currently, studies are underway to examine more closely how these patients differ from those with Bruton's disease.

In addition studies directed at the regulation of T-cell development, we have begun to study the functional and molecular characteristics of human T-lymphocytes expressing the gamma/delta form of the human T-cell antigen receptor in a patient with a unique cellular immunodeficiency associated with an expanded population of morphologically normal gamma/delta T-cells. While appearing to express a single form of the gamma chain, these gamma/delta cells have been found to be

heterogeneous with regard to the cell surface expression of delta molecules, CD2 molecules and CD8 molecules. They are functional in T-cell mediated cytotoxic reactions but fail to proliferate to a variety of stimuli. Studies of this patient's gamma/delta cells as well as gamma/delta cells from normal individuals will hopefully lend insight into the structure and function of the genes being expressed in this lineage of early T-cells and their relationship to T-cells expressing the more common alpha and beta chains of the T-cell antigen receptor.

Publications:

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Marcon L, Fritz ME, Kurman CC, Jenson JC, Nelson DL. Urinary receptors for human interleukin-2. *Clin Exp Immunol* 1988;73:29-33.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04018-13 MET

## PERIOD COVERED

October 1, 1988 - September 30, 1989

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

## Immunoregulatory Glycoproteins Purification and Characterization

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

PI: Andrew V. Muchmore, M.D.	Senior Investigator	MET, NCI
Bibhuti Mishra, M.D.	Clinical Associate	MET, NCI
Neeraja Sathyamoorthy, Ph.D.	Visiting Fellow	MET, NCI
Jean Decker, B.S.	Chemist	MET, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolism Branch

## SECTION

Cellular Immunology Section

## INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

4

## PROFESSIONAL:

4

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews

"B" 100%

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

Last year represented a transition in the major focus of my laboratory. Research has progressed from the purification, immunologic characterization, sequencing, and cloning of uromodulin, an 85 KD immunorepressive glycoprotein derived from pregnancy urine, to focusing on the characterization of glycosylation patterns responsible for the biologic activity of uromodulin. Utilizing a combination of protease digestion and multiple chromatographic steps, we have purified to homogeneity and structurally characterized by <sup>1</sup>H NMR two profoundly biologically active high mannose oligosaccharides. Based on these structural observations, alternative sources for active oligosaccharides have been found which include egg white protein, soybean meal, and yeast mannan. These compounds inhibit and enhance the immune response both *in vitro* and *in vivo*. Utilizing defined oligosaccharides ranging from 7-11 sugar residues, we have performed a number of structure function studies. These compounds inhibit T cell responsiveness, enhance PGE<sub>2</sub> synthesis, induce collagenase release, block hydrogen peroxide formation, activate the hexose monophosphate shunt, bind to IL-1, TNF, and IL-2, and regulate the expression of DR. We have applied for a patent covering the immunoregulatory activity of these structurally unique compounds.



Project DescriptionMajor Findings:

- 1) The immunosuppressive oligosaccharides of uromodulin have been purified and structurally characterized.
- 2) A panel of related oligosaccharides have also been purified and structurally characterized.
- 3) These compounds have been shown to
  - a. block T cell responsiveness in vitro.
  - b. induce a delayed inflammatory response in vitro.
  - c. induce PGE<sub>2</sub> synthesis in vivo.
  - d. compete with binding to IL-1, IL-2 and TNF.
- 4) In vivo studies have shown species restricted activity in mice, guinea pigs and miniature swine.
- 5) In vitro studies now demonstrate that these mannose oligosaccharides directly interact with the transcriptional activator factors AP-1, AP-3 and TNF-1.

We have also shown that IL-2 and TNF directly bind to class II antigens and can regulate their expression in vitro. This binding is mediated by oligosaccharides expressed by class II molecules.

Publications:

Blaese RM, Poplack D, Muchmore AV. Disorders of the mononuclear phagocytic system. In: Stiehm R, Fulgeniti V, eds. Immunologic disorders of infants and children, 3rd Ed. Philadelphia: WB Saunders, 1989;364-83.

Sherblom AP, Decker JM, Muchmore AV. The lectin like interaction between recombinant tumor necrosis factor and uromodulin. J Biol Chem 1988;263:5418-24.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04020-12 MET

## PERIOD COVERED

October 1, 1988 - September 30, 1989

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

Antigen-specific T-cell activation application to vaccines for malaria and AIDS

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

PI: Jay A. Berzofsky, M.D., Ph.D.	Section Chief	MET, NCI
Akihiko Kurata, M.D., Ph.D.	Guest Researcher	MET, NCI
Hideimi Takahashi, M.D., Ph.D.	Visiting Fellow	MET, NCI
Anne Hosmalin, M.D.	Visiting Fellow	MET, NCI
Rosa Toper, Ph.D.	Visiting Fellow	MET, NCI
Judy Kim	Hughes Medical Student	MET, NCI

## COOPERATING UNITS (if any)

Louis H. Miller, M.D.	Chief, Malaria Section	LPD, NIAID
Gene H. Shearer, Ph.D.	Section Chief	EIB, NCI
Robert Gallo, M.D.	Lab Chief	LTCR, NCI

## LAB/BRANCH

Metabolism Branch (More professional personnel listed on next page)

## SECTION

Molecular Immunogenetics and Vaccine Research Section

## INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

11.5

## PROFESSIONAL:

9.5

## OTHER:

2

## CHECK APPROPRIATE BOX(ES)

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

(a1) Minors      "B" 100%

(a2) Interviews

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

We have studied the mechanisms by which T cells recognize antigens that are proteolytically processed and presented in association with major histocompatibility complex (MHC)-encoded molecules, the factors that determine which antigenic structures are immunodominant, and the application of these principles to the design of synthetic vaccines for AIDS and malaria. We identified an intracellular thiol protease like cathepsin B as the major protease necessary and sufficient for processing myoglobin to present multiple determinants, allowing prediction of sites of cleavage. Using allelic MHC molecules and site directed mutants of a class II MHC molecule, we demonstrated that T cell receptors (TCR) interact with MHC molecule residues as well as the antigenic peptide, and that the same determinant can bind to the same MHC molecule in different ways. We showed that both helper and cytotoxic T cells (CTL) tend to recognize sequences that can fold as amphipathic helices, and that such peptides do fold as helices in membrane-mimetic solvents. We found that humans can distinguish variant malaria epitopes, but that cross-reactivity also occurs that may allow a multivalent vaccine. We also demonstrated two protective mechanisms in a mouse malaria model, one requiring CTL, and identified CTL epitopes from mouse and human malaria circumsporozoite proteins. We identified multideterminant regions of the HIV envelope seen by mice of multiple MHC types, as well as peptides seen by 50-67% of humans of diverse HLA types, allowing for the possibility to circumvent MHC restriction problems. We showed that some of these T-cell epitopes could elicit help for antibody responses in mice, goats and monkeys. We found that humans infected with HIV retained IL-2 production in response to soluble protein antigens even when T-proliferative responses were lost. We also showed that HIV sequence variation in an immunodominant CTL determinant involved residues interacting with class I MHC molecules as well as ones interacting with the TCR, and suggested a mechanism by which HIV escapes immune control.

Continuation Sheet for PHS 6040

## Other Professional Personnel:

Anne S. DeGroot, M.D.	Guest Researcher	MET, NCI
Michael Good, M.D., Ph.D.	Guest Researcher	MET, NCI; LPD, NIAID
Hanah Margalit, Ph.D.	Visiting Fellow	LMB, NCI
Marika Kullberg	Guest Researcher	MET, NCI

## Other Cooperating Units:

Ronald N. Germain, M.D., Ph.D.	Section Chief	LI, NIAID
Bernard Moss, M.D., Ph.D.	Lab Chief	LVD, NIAID
Walter Weiss, M.D.	Lt. Cdr.	ID, NMRI
Stephen Hoffman, M.D.	Cdr.	ID, NMRI
Sanjai Kumar	Visiting Fellow	LPD, NIAID
Mario Clerici	Visiting Fellow	EIB, NCI

## Project Description

### Major Findings:

We have been studying the mechanisms by which T cells recognize antigens on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, the factors that determine which antigenic structures are more likely to be recognized, and the application of these principles to the design of synthetic vaccines for AIDS and malaria. T cells recognize antigen after it has been proteolytically processed into fragments or unfolded forms which then associate with MHC molecules. Each of these steps can influence which antigenic determinants are seen by T cells.

At the antigen processing step, we have found that the products of natural processing of antigen are generally larger than the synthetic peptide corresponding to the minimal antigenic determinant, and may contain structures that hinder binding to the MHC molecule or T-cell receptor (TCR). Therefore, it is of importance to be able to predict the structure of processed fragments. As a first step toward prediction of the sites of cleavage, we have analyzed the proteases responsible for processing myoglobin to present three different determinants to different T-cell clones. The same protease, an intracellular thiol protease such as cathepsin B, was both necessary and sufficient to process myoglobin to present all three determinants. Moreover, the sites of cleavage by cathepsin B could account for the sizes of fragments produced by the processing of myoglobin. Therefore, identification of this protease may allow prediction of cleavage sites for other protein antigens, and the structure of their processed fragments.

Second, different MHC molecules preferentially present different peptides from the same protein. However, we have found a case in which the same immunodominant determinant is seen by T cells from three distinct MHC types, in each case with different class II MHC molecules. Studies of overlapping peptides and peptides with amino acid substitutions suggest that the different T cells are seeing the peptide in a similar conformation, with similar critical residues. Nevertheless, none of the T-cell clones responded to the peptide on more than one of the three allelic class II MHC molecules, indicating that T cells were specific for the MHC molecule as well as for the peptide. Either the TCR bound directly to residues of the MHC molecule itself, or the peptide bound to MHC in subtly different conformations not detected by our studies. To determine the role of residues of the MHC molecule more directly, we studied a series of site-directed mutants made by D. McKean containing single amino acid substitutions, or groups of substitutions, that correspond to polymorphic differences between two alleles of this class II MHC molecule. Mutations at certain positions differentially affected presentation of the same antigenic determinant to different T-cell clones. If the mutant residues were on the MHC helices, they could contact either the TCR or the antigenic peptide, but one mutation on the floor of the peptide-binding groove, inaccessible to the TCR, enhanced presentation of the same determinant to some T cells and diminished it to others. This result suggests that different T cells can see the same determinant in different positions or conformations in the same MHC molecule. These results affect attempts to determine which residues of a peptide interact with the TCR or the MHC molecule, and make it unlikely that a common sequence motif can be found that will explain all peptides that bind to a given MHC molecule.



Third, we have examined structural features intrinsic to the antigenic determinant, and found that peptides that can fold as amphipathic helices are statistically more frequent among immunodominant T-cell determinants than in protein sequences in general. With a sampling twice the size of our original study, the fraction of sites with this property that are immunodominant with class II MHC molecules has remained nearly the same (71% vs 75%), and still highly significant ( $p < 0.003$ ). Moreover, only a few sites seen with class I MHC molecules have been identified, but 10/11 of these also have this property, indicating that the same chemical principles probably apply to recognition with class I MHC molecules as well. In collaboration with Lila Gierash, we have also found that peptides with sequences compatible with folding as amphipathic helices form such helices in membrane-mimetic environments as measured by circular dichroism, and are surface active by tensiometry, whereas two control peptides did not have these properties. Finally, we tested the predictive capability of the amphipathicity algorithm by searching for a T-cell epitope in the 1-55 region of myoglobin where we knew one existed but had not been localized. Using evenly spaced overlapping peptides, we identified the major epitope in this sequence, and it corresponded to the segment with the highest amphipathic score. These correlations may be valuable in searching for immunodominant determinants for vaccine development.

We applied these results to the design of synthetic vaccines for malaria and AIDS. In the malaria case, with Lou Miller's malaria section, we had previously found two immunodominant sites for helper T cells of both mice and humans, both predicted by this algorithm, from the P. falciparum circumsporozoite protein. These derive from parts of the protein that are variable among isolates of P. falciparum, so we asked whether this variability affected T-cell recognition. We found that T cells from some humans from a malaria-endemic area of Africa could distinguish the variants of these sites, so that the mutations are not immunologically silent. On the other hand, evidence was obtained for crossreactivity among the variants of both sites, so that it should be possible to develop a vaccine that may be useful for many strains of P. falciparum. In mice, we found that the protective response elicited by irradiated rodent malaria P. yoelii sporozoites was under strong MHC control, and that there were two mechanisms of protection, each under different MHC-linked control. One mechanism involved CD8 T cells and the other did not, but neither correlated with antibody level. In the case where CD8 cells were required for protection, we have isolated cytotoxic T lymphocytes (CTL) and identified the epitope they recognize. We also identified a similar CTL epitope from the circumsporozoite protein of the human malaria P. falciparum.

In the case of AIDS, we have identified 6 multideterminant regions of the envelope protein that contain overlapping or abutting helper T-cell antigenic determinants that are seen by mice of different MHC types, so that each region can be seen by 3/4 or 4/4 MHC types. Five of the six regions are predicted by amphipathicity, and four are from conserved segments of the envelope sequence. Four of the peptides identified in mice were studied in HIV-infected humans. With Gene Shearer's lab, we found that about 80% of humans in early HIV infection lose the ability to respond in a T-cell proliferative response to soluble protein antigens, but about 50% retain a T-cell response to the same antigens as measured by IL-2 production. This finding may be of prognostic significance. Also, we could thus use IL-2 production to test responses of asymptomatic HIV seropositive donors to these peptides. Each peptide elicited in vitro T-cell responses in cells from 50-67% of unrelated HLA-diverse donors who retained the ability to respond to control

antigens, and 85-90% of donors responsive to control antigens could respond to at least one of the four peptides. In collaboration with Bart Haynes and Tom Palker, we found that one of these peptides served as a very potent helper determinant when attached to a neutralizing antibody epitope, so that the construct could elicit antibodies in both goats and mice without a carrier. In goats, the construct with the envelope T-helper determinant elicited higher titers of neutralizing antibodies than a conjugate of the same neutralizing antibody epitope attached to a protein carrier. This same helper determinant also could be used in vivo to prime rhesus monkeys for a subsequent enhanced secondary antibody response on their first exposure to the whole HIV envelope protein.

Finally, because the CTL determinant we previously identified in the envelope protein is highly variable among HIV isolates, we examined the effect of this variability on T-cell recognition in collaboration with the labs of Ron Germain and Bernard Moss. We found that the same site is a CTL determinant on several isolates of HIV, even though the CTL do not crossreact. By studying substitutions at each position, we could identify residues that contribute to binding to class I MHC molecules, and one residue that seems critical for recognition by the TCR. Thus HIV can mutate residues that interact with MHC molecules. Because a) we have found that sites on HIV proteins that are presented by class I MHC molecules are more rare than those presented by class II molecules, b) it is known that CD8 CTL can suppress replication of the virus, and c) it is known that HIV can mutate during the course of disease of a single individual, we propose that such mutations in critical sites that prevent binding to MHC molecules of the infected individual may allow the virus to become partially invisible to CTL of that person, escape control, and cause AIDS. The identification of these helper and cytotoxic T-cell determinants, especially ones recognized by individuals of many MHC types, and the understanding of the function of virus variability, should prove useful in the design of vaccines.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
 Z01 CB-04024-2 MET

PERIOD COVERED

October 1, 1988 - September 30, 1989

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

Control of Gene Expression in Lymphoid Development

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

PI:	Louis M. Staudt, M.D., Ph.D.	Senior Staff Fellow	MET, NCI
	Patricia Fast, M.D.	Medical Staff Fellow	MET, NCI
	Hon-Sum Ko, M.D.	Fogarty Visiting Fellow	MET, NCI
	Mitchell Rosner	Guest Researcher	MET, NCI
	Alessandra Vigano, Ph.D.	Fogarty Visiting Fellow	MET, NCI
	Wayne Tsang, M.D.	Medical Staff Fellow	MET, NCI
	Sharon Doll, Ph.D.	IRTA Fellow	MET, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

7

PROFESSIONAL:

5

OTHER:

2

CHECK APPROPRIATE BOX(ES)

(a) Human subjects       (b) Human tissues       (c) Neither      "B" 100%

(a1) Minors

(a2) Interviews

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

As an approach to understanding development in the B lymphocyte lineage, we have characterized trans-acting factors that mediate lymphoid-specific gene transcription. As a model we have studied the lymphoid-specific transcription of immunoglobulin (Ig) genes. The promoters of all Ig variable genes and the Ig heavy chain enhancer contain a critical octamer DNA motif, ATTTGGAT. This octamer motif, together with a TATA motif, is necessary and sufficient for lymphoid-specific promoter activity. Two major nuclear proteins bind the octamer motif specifically: a non-tissue-specific protein (Oct-1) and a lymphoid-specific protein (Oct-2). We cloned the human Oct-2 structural gene by screening a phage  $\lambda$ gt11 expression library with a radiolabeled DNA probe containing the octamer motif. The cloned gene is itself lymphoid-restricted in expression. By sequencing the Oct-2 gene, we identified a 60 amino acid domain that is homologous to the "homeo box" domain of proteins which are important for cell type determination and development in lower eukaryotes. This domain of the Oct-2 protein was shown to be required for DNA binding by site directed mutagenesis. This represents the first demonstration that a mammalian gene containing a homeo box domain is a sequence-specific DNA binding protein. The presence of the homeo box in the Oct-2 gene lends support to the notion that this gene is important for lymphoid development. Full length Oct-2 cDNA clones revealed additional regions of homology with other transcription factors. One highly conserved 75 amino acid domain, the "POU" box, is shared by a subset of homeo box proteins. In addition, Oct-2 has a "leucine zipper" domain which it shares with the nuclear oncogenes myc, fos and jun and with other transcription factors. The leucine zipper may mediate the interaction of Oct-2 with other regulatory proteins. Alternative splicing of the Oct-2 gene generates a variety of distinct Oct-2 proteins, one of which lacks the leucine zipper and thus might be functionally unique.

Project DescriptionMajor Findings:Cloning of a gene (oct-2) which encodes a lymphoid-restricted protein that binds to the immunoglobulin octamer DNA motif.

Immunoglobulin (Ig) genes are expressed only in lymphoid cells and this expression depends on a regulatory octamer DNA motif, ATTTGCAT, found in Ig promoters and in the Ig heavy chain enhancer. At least two nuclear proteins bind the octamer motif specifically: Oct-1, which is found in all cell types, and Oct-2, which is restricted to lymphoid cells. Oct-2 most likely mediates the lymphoid-specific transcriptional activity of Ig promoters. In order to understand this transcriptional activation on a molecular level, we decided to clone the gene encoding Oct-2. We used a new method of screening expression libraries in phage  $\lambda$ gt11 with radiolabeled DNA probes containing the octamer motif. An important modification of the protocol made the cloning possible: DNA probes were used that were tandem repeats of the octamer motif. These multimer probes gave readily detectable signals from phage plaques that could not be detected using probes containing only one copy of the octamer motif. The two recombinant phage isolated by this procedure contained cDNA inserts encoding proteins that bound specifically to the octamer motif. The cDNA inserts of these two phage crosshybridized with each other and define what we term the oct-2 gene. The DNA binding characteristics of the oct-2 gene product and Oct-2 were virtually identical. Furthermore, oct-2 was found to be expressed predominantly in lymphoid cells. The amount of oct-2 mRNA expressed in a given lymphoid cell line correlated well with the amount of Oct-2 found in the same cell line. Pre-B cell lines showed a coordinate increase in oct-2 mRNA and Oct-2 protein upon stimulation with bacterial lipopolysaccharide. Given these correlations, the oct-2 gene most likely encodes Oct-2.

The oct-2 gene contains a functional homeo box domain

The two oct-2 partial cDNAs were sequenced and found to be identical in their overlap. The predicted oct-2 protein sequence was compared to all possible translations of the GenBank Genetic Sequence Data Bank and similarities were seen with proteins containing the so-called homeo box domain. The homeo box is a 60 amino acid domain which was originally found in many developmentally important Drosophila proteins and in yeast mating type proteins that control gene expression in a cell type-specific fashion. These homeo box proteins have been shown to be sequence-specific DNA binding proteins which act as "master switches" by controlling gene expression and thus cellular differentiation. Human and mouse genes have been identified which contain homeo boxes but the function of these genes is unknown. The oct-2 sequence showed that it is evolutionarily divergent from the previously cloned human homeo box genes and, furthermore, it is located on a different human chromosome (#19). Site directed mutation of the homeo box domain destroyed binding to the octamer motif, thus demonstrating for the first time that a human homeo box protein is a sequence specific DNA binding protein. We noted that the binding sites of the yeast and Drosophila homeo box proteins were similar to the Ig octamer motif and showed that the oct-2 protein also bound to these sequences. Thus, all homeo box proteins may have a binding preference for A-T rich, Ig octamer-like sequences. In summary, it is tempting to speculate that the oct-2 gene product, on the basis of its homeo box homology, may play an important



role in development in the lymphoid system.

#### Oct-2 shares additional domains with other transcription factors

A full length cDNA clone encoding the entire Oct-2 coding region was cloned from a human activated B cell library using the partial oct-2 cDNA as a probe. This cDNA clone was transcribed and translated in vitro and was shown to encode an octamer binding protein with an apparent molecular weight of approximately 60 kD, the molecular weight of Oct-2 purified from B cell nuclear extracts. The predicted amino acid sequence of Oct-2 contains a 75 amino acid domain that is found in a subset of homeobox proteins. This domain, termed the "POU" domain, is shared by Pit-1, a human pituitary-specific transcription factor, Oct-1 and Oct-2, and Unc86, a gene involved in central nervous system development in the nematode, *C. elegans*. The POUbox family of proteins have homeodomains that are more related to each other than to other homeobox proteins, thus suggesting that they represent an evolutionarily divergent subset of the homeobox family. Partial Oct-2 proteins which lack the POU domain still interact with the octamer motif specifically, albeit with lower affinity, suggesting that the POU domain may be involved primarily in other aspects of the function of this transcription factor besides DNA binding. The amino terminal region of Oct-2 is highly glutamine rich, a feature which it shares with a variety of other eukaryotic transcription factors. In the human transcription factor SPL, the glutamine-rich domain is essential for transcriptional activation but not DNA binding, and it may be functioning analogously in Oct-2. Finally, Oct-2 contains in its carboxy terminus a so-called "leucine zipper", a potentially alpha helical region in which every seventh amino acid is a leucine. Leucine zippers have been found in other transcription factors and in the oncogenes fos, jun and myc and are responsible for homodimerization, heterodimerization and oligomerization of these proteins. Thus, the Oct-2 leucine zipper may be involved in protein-protein interactions which distinguish its activity from that of other octamer-binding proteins such as Oct-1.

#### Alternative splicing creates structurally distinct Oct-2 proteins

Previous Northern blot analysis of Oct-2 mRNA revealed at least 7 distinct mRNA sizes in B lymphocytes, ranging from 7.2 kilobases (kb) to 1.3 kb. By cloning and sequencing multiple Oct-2 cDNAs from an activated B cell library, we found that some of this size heterogeneity results from alternative splicing of the Oct-2 mRNA. We have so far found four Oct-2 mRNAs with alternative splices in the coding region. One particularly interesting alternative splice removes most of the leucine zipper domain. The functional consequences of these alternative splicing are being investigated. In order to look for possible size heterogeneity in the Oct-2 proteins found in nuclear extracts of B cells, rabbit antisera were prepared against bacterially expressed Oct-2 or against synthetic peptides derived from Oct-2. In Western blots of nuclear extracts, these antisera reacted with a family of four proteins ranging from 58kD to 61kD which were only found in B lymphocytes. Thus, the alternative splicing that we observed among the oct-2 cDNAs may result in heterogeneity in Oct-2 proteins found in the nucleus of B cells.



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SUMMARY REPORT  
IMMUNOLOGY BRANCH  
October 1988 - September 1989

The Immunology Branch carries out laboratory investigations in basic immunology with particular emphasis on studies of the major histocompatibility complex (MHC) and its role in transplantation in animal models and in man. The major areas of study are as follows: 1) Immunogenetics of the murine MHC; 2) Development of models for the induction of specific tolerance to MHC products; 3) Genetics of the miniature swine MHC; 4) Molecular biology of porcine class II genes; and 5) Studies of bone marrow and organ transplantation in miniature swine. This report summarizes research efforts in each of these areas during the past year, with reference to the individual annual reports in which details can be found.

1. IMMUNOGENETICS OF THE MURINE MHC

A breeding program is carried out for the maintenance of pedigreed inbred and congenic resistant strains of mice and for the development of new recombinant H-2 haplotypes of value for structural and functional studies of genes within the MHC (5023). This program also includes the production of monoclonal antibodies reactive with products of MHC genes. New strains bearing recombinant MHC haplotypes (of which 22 have been derived from the breeding program of the Immunology Branch) are utilized for the production of new reagents and probes to study the fine structure of the MHC.

A new recombinant derived from this program, the A.TH-Q1b, has made possible for the first time an analysis of the A.TH anti-A.TL reactivity in the absence of a Qa-1 difference. Skin grafts between the new A.TH-Q1b animals and ATL were found to be markedly prolonged, indicating that class II differences alone may be insufficient to cause prompt skin graft rejection. These data may clarify the previously puzzling finding that some class II antigens cause skin graft rejection while others do not. The data suggest that one role of class II differences in skin graft rejection may be the presentation of other minor antigenic differences to the immune system.

2. DEVELOPMENT OF MODELS FOR THE INDUCTION OF SPECIFIC TOLERANCE TO MHC PRODUCTS

The induction of intentional mixed chimerism across MHC barriers has been investigated as a means of producing specific transplant tolerance (5021). The use of mixtures of T-cell depleted syngeneic plus allogeneic bone marrow has been found to produce permanent, specific tolerance with full immunocompetence. Reconstitution of irradiated animals with a mixture containing T cell depleted syngeneic marrow plus non-T-cell depleted allogeneic marrow has been found to produce complete allogeneic chimerism while avoiding GVHD. This model has been found to have potential usefulness in the treatment of leukemia, since the procedure does not appear to eliminate the graft-vs-leukemia (GVL) effect.

In order to decrease the toxicity of ablative radiation regimens used to prepare radiation chimeras, the use of monoclonal anti-T-cell subset antibodies as a substitute for part of the irradiation has been explored. Treatment with such antibodies in vivo has been found to deplete mature T-cells effectively from all

peripheral lymphoid tissues, but not from the thymus. Selective thymic irradiation has therefore been added to this regimen, and has produced long-term mixed chimerism and specific tolerance across an MHC barrier with a non-lethal preparative regimen. This regimen has now been successfully applied to rat-mouse xenogeneic chimeras as well.

### 3. GENETICS OF THE MINIATURE SWINE MHC

Partially inbred strains of miniature swine, NIH mini-pigs, have been developed over the past fifteen years by this laboratory as a large animal pre-clinical model for transplantation studies. Five new recombinant haplotypes have been identified within these herds, and three of these have been bred to homozygosity. By serologic, biochemical and genetic data all of these recombinants appear to have split class I from class II loci.

Using the new recombinant MHC haplotypes, the relative importance of class I versus class II antigens for allograft survival has been examined. Both class I and class II antigens were found sufficient to produce prompt rejection of skin. However, class II antigen matching appeared to be of overwhelming importance in determining the outcome of vascular allografts such as kidney transplants (5023). The mechanism of tolerance induced by class I mismatched kidneys has been assessed by culturing cells from the transplants.

### 4. MOLECULAR BIOLOGY OF PORCINE CLASS II GENES

Because of the importance of class II antigens to renal allograft survival, a molecular approach to the analysis of class II genes in this species has been initiated (5023). DNA from each of the independent MHC haplotypes has been examined by extensive RFLP analysis using probes for human class II  $\alpha$  and  $\beta$  genes. Hybridization with the human DQ $\alpha$  cDNA probe revealed a single fragment in each of the enzymatic digests performed, and showed limited but definite polymorphism. Hybridization with the human DR $\alpha$  cDNA probe showed a set of bands clearly distinct from those revealed with DQ $\alpha$ , and showed no polymorphism among the herds. In contrast to the unique hybridization patterns of class II  $\alpha$  probes, hybridization with class II  $\beta$  probes showed extensive cross hybridization. Polymorphism between all three haplotypes was apparent in many of the digests.

A genomic library has been constructed from the SLA<sup>C</sup> lymphocyte DNA, and cDNA libraries have been prepared from SLA<sup>C</sup> and SLA<sup>D</sup> spleen message. These libraries have been screened with human class II cDNA probes, and clones analogous to many of the human class II  $\alpha$  and  $\beta$  genes have been identified. Sequences of the cDNA clones have been carried out, as have studies of expression following transformation into mouse cell lines.

### 5. STUDIES OF BONE MARROW AND ORGAN TRANSPLANTATION IN MINIATURE SWINE

Without exogenous immunosuppression, miniature swine matched for class II and transplanted across a class I plus minor antigen difference have been found to frequently develop specific systemic tolerance. The mechanism of this tolerance has been investigated in vitro (5023) and has been found to involve a specific depletion of class I reactive helper T-cell populations. Also, consistent with a lack of T-cell help is the fact that recipients of class II matched renal allografts which went on to accept such grafts long-term showed a brief rejection

crisis at approximately fourteen days during which there was a rise in IgM antibodies to SLA class I antigens, which subsided spontaneously without conversion from IgM to IgG.

Because the response to ablative regimens in miniature swine is much more similar to that of man than is the response of rodents, establishment of a bone marrow transplant regimen in this model has been carried out as a pre-clinical step (5023). Ablative radiation regimens have been established similar to those used for HLA identical sibling transplants in man, and have been found satisfactory to permit MHC matched bone marrow allografts. A new regimen with fractionated irradiation has been found necessary to permit successful transplantation of MHC mismatched bone marrow. This regimen has been found not sufficient to permit T-cell depleted bone marrow grafts to be accepted. Non-T-cell depleted bone marrow grafts across full MHC barriers were found to produce aggressive lethal GVHD. Addition of T-cell depleted autologous bone marrow to the non-T-cell depleted allogeneic inoculum has been attempted and, in some cases, has permitted engraftment while avoiding lethal GVHD. These data may have potential applicability to clinical transplantation.

The use of bone marrow transplants to induce tolerance to organ allografts in this model have been demonstrated. Kidney grafts matched for MHC with the bone marrow donor have been found to enjoy the same long-term survival as do matched kidney allografts.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05021-18 I

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Antigens Determined by the Murine Major Histocompatibility Locus

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

PI: D. H. Sachs Chief, Transplantation Biology Section IB, NCI

Others: T. M. Sundt III Medical Staff Fellow IB, NCI  
 Y. Sharabi Visiting Fellow IB, NCI  
 M. Sykes Senior Staff Fellow IB, NCI  
 Z. Bukhari Visiting Fellow IB, NCI  
 I. Aksentijevich Biotechnology Fellow IB, NCI

## COOPERATING UNITS (if any)

S. L. Epstein, Senior Staff Fellow, National Center for Drugs and Biologics, FDA

## LAB/BRANCH

Immunology Branch

## SECTION

Transplantation Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

4.0

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

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

B

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

Studies are being directed toward understanding the major histocompatibility complex, the structure and function of the products of this complex, and manipulations of immune responses to these products. Current studies include: 1) Characterization of major histocompatibility antigens: Congenic resistant strains of mice are developed, maintained, and used in serologic and immunochemical analyses of the MHC products of the mouse; 2) Studies of monoclonal antibodies to H-2 and Ia antigens: Hybridoma cell lines are produced by fusion of immune mouse spleen cells with mouse myeloma cells. The monoclonal anti-H-2 and anti-Ia antibodies produced by these hybridomas are analyzed by serologic and immunochemical means and are used to further characterize the fine structure of the MHC; 3) Characterization of receptor sites for histocompatibility antigens: Anti-idiotypic antisera are produced against anti-H-2 and anti-Ia hybridoma antibodies, and the effects of these antisera on in vitro and in vivo parameters of histocompatibility are assessed; 4) Mechanism of tolerance to H-2 and Ia antigens: The humoral and cellular responses of radiation bone marrow chimeras are examined, and the mechanism for maintenance of tolerance in these animals is studied; 5) Mixed allogeneic and xenogeneic chimeras: Irradiated animals are reconstituted with mixtures of T-cell depleted donor and host marrow, and the mechanism of tolerance and of immune responsiveness in these animals is studied in vivo and in vitro; 6) Non-lethal preparative regimens for bone marrow transplantation: Attempts to replace lethal irradiation with monoclonal antibody treatment in vivo are examined, and the immune status of the resulting animals is studied; and 7) Graft-vs-leukemia (GVL) effects of mixed bone marrow reconstitution: The effects of reconstitution with T-cell depleted syngeneic plus non-T-cell depleted allogeneic bone marrow on syngeneic leukemia are examined.

## Project Description

Major Findings: 1) Fusion of spleen cells from mice hyperimmunized against H-2 antigens and boosted 2-3 days before fusion has been found to give satisfactory results in the production of anti-H-2 hybridomas. About 70 stable hybridomas have so far been produced, most of which detect H-2 or Ia antigens of a variety of haplotypes. Panel testing has indicated that most of these antibodies react with public specificities of the H-2 and Ia antigens, while a few appear to detect private specificities. Numerous crossreactions have been detected using these monoclonal antibodies, which define a variety of new public H-2 and Ia specificities.

2) Using a new MHC recombinant strain (C3H.KBR) we have detected an unusually high responsiveness to Qa antigens. Hybridomas prepared using appropriately immunized spleen cells from this strain yielded 11 new monoclonal antibodies directed against several epitopes of the Qa antigens. Immunoprecipitation studies using these antibodies indicate that at least two different molecules are detectable. In vivo injection of these antibodies has also been found to have profound effects on circulating T-cells. Two of these antibodies of the same immunoglobulin class but directed toward different epitopes of Qa-2 have been found to have different in vivo T-cell depletion characteristics. In vitro studies show that the antibody causing rapid depletion of T-cells in vivo causes much less cell surface modulation of Qa-2 than does the antibody leading to slow depletion. This difference may indicate that the antibody causing modulation reacts with the site on Qa-2 involved in its physiologic ligand binding.

3) A new recombinant which occurred during the backcrossing of A.TH has permitted, for the first time, an analysis of A.TH anti-A.TL reactivity in the absence of a Qa-1 antigen difference. Our results with skin grafting experiments indicate that in the absence of a Qa-1 difference, there is marked prolongation of skin graft survival. These studies suggest that a class II difference alone may be insufficient to cause skin graft rejection, and that class II antigens may actually function by presenting other antigenic differences (e.g., minor antigens or Qa antigens) to the immune system.

4) Mice reconstituted with mixtures of syngeneic plus either allogeneic or xenogeneic bone marrow have been found to manifest specific hyporeactivity to donor type skin grafts at 8 weeks following reconstitution. In the case of mixed allogeneic reconstituted animals, both host and donor lymphoid elements are found in the peripheral circulation, while only host elements can be found in the mixed xenogeneic reconstituted animals. In vitro assays have been performed in order to examine the mechanism of transplantation tolerance in these animals. Evidence for generation of high levels of natural suppressor (NS) cells in the early weeks following grafting has been obtained. Characterization of these suppressor cells by antibody reactivity indicates that they are not T cells. They are derived predominantly from the host component of mixed marrow grafts and may be involved in the elimination of those alloreactive cells which otherwise would cause GVH.

5) Because in vitro assays using spleen cells from mixed chimeras showed maximal suppressive activity at approximately 8 days following radiation and bone marrow reconstitution, delayed administration of non-T-cell-depleted allogeneic marrow to animals irradiated and reconstituted with T-cell-depleted syngeneic marrow was

performed. This procedure was found to markedly reduce the GVH produced by allogeneic T cells and to result in long-term completely allogeneic chimeras.

6) Elimination of T cells from the syngeneic component, but not the allogeneic component of a mixed bone marrow inoculum has been found to produce completely allogeneic chimeras which are, however, protected from lethal GVHD. By adding the EL4 murine leukemia to the syngeneic component of such a reconstituting inoculum, it has been found that this procedure does not eliminate the strong graft-vs-leukemia (GVL) effect of non-T-cell depleted allogeneic bone marrow. The GVL effect has also been seen when non-T-cell depleted allogeneic marrow was added as late as 8 days following the T-cell depleted syngeneic marrow plus leukemia.

7) Using a mixture of anti-CD4 plus anti-CD8 monoclonal antibodies in vivo, transient bone marrow engraftment of allogeneic bone marrow was achieved with a total body irradiation dose as low as 300R. Addition of 700R thymic irradiation to this regimen led to long-term mixed chimerism and allogeneic skin graft tolerance. Addition of anti-Thy.1 and anti-NK1.1 antibodies to the preparative regimen has permitted extension of this model to rat-mouse xenografts.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05023-18 I

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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:	T. M. Sundt III	Medical Staff Fellow	IB, NCI
	P. C. Guzzetta	IPA Investigator	IB, NCI
	B. R. Rosengard	Medical Staff Fellow	IB, NCI
	C. LeGuern	Expert	IB, NCI
	G. E. Freeman	Biotechnology Fellow	IB, NCI
	K. Nakajima	Visiting Fellow	IB, NCI

## COOPERATING UNITS (if any)

NIH Animal Center, Poolesville, Maryland  
 J. K. Lunney, Research Chemist, USDA Animal Parasitology Institute, Beltsville, MD  
 S. A. Rosenberg, Chief, Surgery Branch, NCI

## LAB/BRANCH

Immunology Branch

## SECTION

Transplantation Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

4.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

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

B

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

Three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. Current projects include: 1) Assessment of survival of organs and tissue transplants among and between members of these herds as a model for human transplantation; 2) Assessment of the immunologic parameters involved in tolerance to allografts in this species; 3) Detection and characterization of intra-MHC recombinants: Five intra-MHC recombinants have been obtained and have been or are being bred to homozygosity. Kidney transplants utilizing these new recombinants have shown that selective matching for Class II antigens frequently permits long-term kidney graft survival across a Class I difference; 4) Bone marrow transplants in miniature swine: the effect of mixing autologous plus allogeneic marrow in the reconstituting inoculum are being examined. This modality is being assessed as a specific preparative regimen for allogeneic organ transplantation; 5) Production and characterization of monoclonal antibodies reactive with subsets of pig lymphocytes: antibodies corresponding to many of the OKT series in man have been identified (including T4, T8, and T11). The effects of these antibodies on in vitro and in vivo transplantation immunity are being assessed, and they are also being used to assess mechanism of tolerance; and 6) Analysis of class II MHC genes: Southern blot analyses using cDNA probes from human class II genes have been performed and indicate that genes corresponding to each of the major human class II loci are present in the pig genome. In addition, a genomic library and two cDNA libraries have been constructed and screened with these probes. Class II genes from the pig herds have been isolated and characterized by hybridization and by sequence analysis. These genes have also been used in vitro and in vivo for transfection studies.

## Project Description

Major Findings: 1) Breeding of further generations has continued to be successful. We therefore now have three different herds of swine, each homozygous for a different SLA antigen, as well as five lines bearing intra-MHC recombinants.

2) Transplants of kidneys between recombinant and non-recombinant haplotypes have permitted an evaluation of the effects of selective class I and/or class II matching on vascular allograft survival. A non-MHC-linked gene determining immune responsiveness has been detected and determined to control rejection of vascular grafts across non-MHC differences. In animals bearing the non-rejector gene at this locus, vascular grafts are accepted across minor differences and also across class I plus minor differences, but not across class II differences. These studies indicate, therefore, that class II matching is of major importance for survival of vascular grafts.

3) Skin grafts are promptly rejected across class I or class II differences. However, following successful kidney transplants in the class II matched situation, donor skin grafts are specifically prolonged, indicating that acceptance of the vascular graft induces systemic tolerance.

4) The mechanism of systemic tolerance in class II matched kidney allografts has been examined. Studies on the generation of CTLs in vitro indicate that such tolerant animals cannot generate a CTL response across a class I only difference, as can 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. In addition, non-T suppressor cells have been identified as contributing to the defect. The role of local suppressor cells is suggested by studies of graft infiltrating lymphocytes.

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  $\alpha$  chain gene corresponding to that of the human DR  $\alpha$  chain, DQ  $\alpha$  chain, and DP  $\alpha$  chain. Of these, only the DQ  $\alpha$  chain appears to be polymorphic within these herds. Approximately 5-7 class II  $\beta$  chain genes have been revealed by these analyses, which show both polymorphism and extensive cross-hybridization between  $\beta$  chains of the three major loci.

6) A complete EMBL-3 phage library has been constructed from the c haplotype, and class II genes corresponding to those detected in the Southern blot analysis have been isolated and characterized.

7) cDNA libraries from the c and d haplotypes have been constructed and screened with human class II probes. cDNA clones corresponding to DQ $\alpha^c$ , DQ $\beta^c$ , DQ $\alpha^d$ , DQ $\beta^d$ , DR $\alpha^d$ , and DR $\beta^d$  have been isolated. Several of these genes have been successfully transfected into mouse L cells, leading to cell surface expression of the swine class II antigens on these cell lines.

8) Analysis of the derived amino acid sequence of the SLA DR $\beta^d$  cDNA gene has led to a surprising finding. Comparison of this sequence with sequences of a panel of human DR $\beta$  genes indicates that at the amino acid level all three hypervariable

regions of the swine sequence are more similar to the corresponding regions of the human DRI $\beta$  allele than are the sequences of any other human DR $\beta$  alleles. At the DNA level, silent substitutions show no differences between different human  $\beta$  alleles. These data therefore suggest a high level of selection leading to the amino acid sequence homology between these species.

9) A series of monoclonal antibodies reactive with a variety of swine lymphocyte surface antigens have been prepared. One of these antibodies recognizes an SLA antigen of the SLAdd haplotype, at least two others react with selective T-cell subpopulations, and at least one antibody is reactive with macrophages. Further characterization of these antibody reactivities is in progress.

10) A protocol which permits bone marrow engraftment across MHC differences has been obtained. Using this protocol, fully allogeneic bone marrow grafts led to lethal GVHD. Using a mixture of T-cell depleted autologous bone marrow plus non-T-cell depleted fully MHC mismatched bone marrow, alloengraftment has been obtained in 17 of 19 animals to date. In 9 of these animals clinical GVHD was self-limited, with resolution by the third week. These data indicate that, as in the murine model, T-cell depleted autologous bone marrow has an anti-GVHD effect when administered as part of the mixed bone marrow reconstitution inoculum. The longest survivor to date has lived 154 days, dying of probable radiation damage to the lungs.

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SUMMARY REPORT  
EXPERIMENTAL IMMUNOLOGY BRANCH  
October 1988 - September 1989

The Experimental Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis in the following areas: 1) lymphocyte differentiation and regulation; 2) cell biology of the immune responses; 3) transplantation biology; 4) structure, regulation and function of genes involved in the immune response; 5) tumor immunology; and 6) flow cytometry. This report briefly summarizes research efforts in each of the foregoing areas during the past year. More detailed information on specific accomplishments can be found in the individual annual reports cited by number in the text.

1. LYMPHOCYTE DIFFERENTIATION AND REGULATION

Dr. Alfred Singer's laboratory has examined the role of CD4 and CD8 accessory molecules in T cell differentiation and function. These studies have revealed that protein kinase C (PKC) activation results in the rapid internalization from the cell surface of TcR and CD4, but does not result in the rapid internalization of CD8, suggesting that the CD4 and CD8 differ in their biochemical responsiveness as well as their ligand specificities for class II and class I MHC respectively (9268). Studies with anti-CD4 mAb modulation of cell surface CD4 have revealed that anti-CD4 induced internalization of CD4 induces co-internalization of TcR both in vitro and in vivo (9268). Interestingly, the co-internalization of TcR with CD4 is dependent either on PKC or a related intra-cellular mediator, as intra-cellular depletion of PKC disrupts the co-internalization of TcR induced by anti-CD4 cross-linking (9268). During these studies, Dr. Alfred Singer's laboratory found that antibody induced engagement of CD4 on immature CD4<sup>+</sup>8<sup>+</sup> (double positive) thymocytes caused the immature double positive thymocytes to markedly increase their expression of TcR by 3-5 fold (9268,9273,9255), as opposed to the reduction in TcR expression that occurs upon antibody induced CD4 engagement on mature CD4<sup>+</sup>8<sup>-</sup> (single positive) thymocytes. Since increased TcR expression is a necessary step in the differentiation of immature double positive thymocytes into mature single positive thymocytes, these studies indicated a critical role in T cell differentiation for CD4 engagement on developing thymocytes (9268,9273). Dr. Singer's studies to elucidate the molecular basis for the increased expression of T cell receptor on immature thymocytes in response to CD4 engagement revealed that the great majority of receptor proteins are degraded within immature thymocytes, and that CD4 engagement results in a decreased rate of degradation (9268,9273). Thus, the increase in TcR expression does not result from increased synthesis, but rather from decreased degradation.

Dr. Alfred Singer's laboratory has also examined the role of T cell receptor expression in thymocyte development. In studies in mice which are genetically unable to express T cell receptor (SCID mice), Dr. Singer's laboratory observed that TcR<sup>-</sup> SCID thymocytes were arrested at the CD4<sup>-</sup>8<sup>-</sup> stage of differentiation. Interestingly, if TcR<sup>+</sup> cells were introduced into the SCID thymus, the SCID thymocytes would remain TcR<sup>-</sup> but would differentiate into CD4<sup>+</sup>8<sup>+</sup> cells (9273,9255). These studies indicated a role for functional TcR<sup>+</sup> cells in initiating the differentiation of TcR<sup>-</sup> CD4<sup>-</sup>8<sup>-</sup> thymocytes, a function that might be performed by TcR( $\gamma\delta$ ) in normal T cell ontogeny.

If thymic selection occurs at the CD4<sup>+</sup>8<sup>+</sup> double positive stage of differentiation, immature double positive thymocytes must be responsive to membrane-initiated differentiation signals. Studies from Dr. Alfred Singer's laboratory have revealed that double positive thymocytes will activate tyrosine kinases which tyrosine phosphorylate the zeta chain of the T cell receptor complex in response to cross-linking of cell surface CD3, CD4, CD8, and TcR( $\alpha\beta$ ) (9273). Since most double positive thymocytes have their zeta chain phosphorylated *in vivo*, these results suggest that most double positive thymocytes have already received *in situ* membrane-initiated differentiation signals that have activated their intracellular tyrosine kinases.

Studies in the laboratory of Dr. Stephen Shaw have been directed at characterization of heterogeneity among subsets of human peripheral blood T cells and determining the functional capacities of the subsets defined (9257, 9255). Their studies confirm and extend previous analysis of T cell subsets distinguished by different isoforms of CD45, which that laboratory has interpreted to be naive and memory T cells. Methodologic progress allows preparation of very pure subsets whose functional capacities have been studied. Naive cells have markedly reduced proliferative responses to various regimens of receptor-mediated activation; however, their capacity to respond is demonstrated by augmented response in the presence of co-stimuli such as that provided by IL1/IL6, CD28 or CD44. This suggests important regulatory mechanisms for costimuli *in vivo* in determining responsiveness of particular subsets. Systematic phenotypic analysis of CD4<sup>+</sup> cells with more than 200 monoclonal antibodies reveals unexpected heterogeneity which is being categorized by multicolor flow cytometry. Such integrated phenotypic and functional analysis is essential to adequately address the functional complexities in T cell responses.

The role of the T cell receptor (TCR)  $\alpha\beta$  heterodimer in antigen recognition has been analyzed in studies carried out in the laboratory of Dr. Richard Hodes (9265). T cell clones specific either for alloantigen or for conventional peptide antigens in association with self major histocompatibility complex (MHC) determinants were analyzed. When the crossreactive repertoires of these clones were studied, a strong and selective association was observed such that most clones specific for the peptide 81-104 of cytochrome c were crossreactive to the Mls<sup>c</sup> alloantigen. An evaluation of TCR usage in panels of clones as well as heterogeneous T cells revealed that the basis for this specificity lies in the fact that expression of the V $\beta$ 3 gene segment is sufficient to confer Mls<sup>c</sup> specificity on a T cell. In addition, it was demonstrated that mouse strains which express Mls<sup>c</sup> delete expression of V $\beta$ 3 in their mature peripheral T cell populations. This failure to express V $\beta$ 3 presumably reflects a consequence of the maintenance of tolerance to self Mls<sup>c</sup> products. These findings provide a basis for the understanding of high T cell precursor frequency for Mls<sup>c</sup> products, reflecting the overall expression of V $\beta$ 3 in the T cell repertoire. Moreover, they demonstrate the importance of these products in selection of the antigen-specific T cell repertoire through a mechanism which appears to involve the clonal deletion of potentially self-reactive T cells. An analysis of 1) the extent of TCR V $\beta$  deletions which occur in generation of the mature TCR repertoire, and 2) the range of self determinants which play a role in these TCR deletions was carried out by studying mRNA corresponding to 16 V $\beta$  gene products in a large panel of inbred strains. Strain-specific deletions in 9 of the 16 V $\beta$  families were detected and were shown to be related to the expression of

multiple MHC and non-MHC self determinants. These findings indicate that maintenance of tolerance to a variety of self determinants results in substantial deletions in available TCR V $\beta$  repertoire (9265). The role of the thymus in TCR negative selection was analyzed by examining TCR V $\beta$  expression in T cells which have matured in congenitally athymic nude mice. It was found that V $\beta$  deletions which normally occur in mice expressing MHC class II E $\alpha$ E $\beta$  determinants (V $\beta$ 11) or non-MHC MIs<sup>c</sup> determinants (V $\beta$ 3) fail to occur in the CD4<sup>+</sup> and CD8<sup>+</sup> T cells of athymic mice, demonstrating that efficient negative selection is thymus-dependent (9265).

The role of endogenous lymphokines in T cell activation has also been evaluated in Dr. Hodes' laboratory (9205). The proliferative responses of type 2 T helper clones are inhibited by antibody specific for the lymphokine interleukin 4 (IL4), which is produced by these clones. This demonstrates that IL4 acts as an autocrine growth factor for these T cells. In addition, mRNA levels corresponding to a number of activation genes are inhibited by the presence of anti-IL4 antibody during T cell activation. In contrast, the level of IL4 mRNA actually increases in the presence of this antibody, suggesting that endogenously produced IL4 may normally down regulate its own steady state mRNA levels.

Dr. Gene Shearer's laboratory has investigated a series of murine models of autoimmunity with lupus-like characteristics, including a graft versus host (GVH)-induced model of lupus, the genetically determined lupus-like syndrome in MRL/lpr mice, and a retroviral induced model of B cell lymphoproliferative disease (9282). All three models are characterized by autoantibody production and activation of suppressor T cells that are selective in their effect on CD4<sup>+</sup> T helper cells. Preliminary studies in humans with SLE indicate that most of these patients exhibit a selective defect in CD4<sup>+</sup> T helper function. These studies may provide a better understanding of immune regulation and dysregulation in autoimmune disease.

Dr. Shearer's laboratory has also investigated the effects of different immunologic insults on the development of murine T lymphocytes from bone marrow stem cells (9264,9255). These studies indicate that either a GVH reaction or treatment of mice with cyclosporin A (CsA) results in thymic damage such that after irradiation and bone marrow grafting, both CD4<sup>+</sup> and CD8<sup>+</sup> cells develop, but there is a selective absence of CD4<sup>+</sup> T helper function. If bone marrow transplantation precedes treatment with CsA, mice fail to develop single positive (CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>+</sup>CD8<sup>+</sup>) T cells. These results provide a model that should be useful for investigating: a) the role of the thymus in T cell development; b) the expression of T cell receptors; and c) the effects of GVH and CsA on immune reconstitution in patients exposed to these immunologic insults.

Studies of T lymphocyte responses in HIV<sup>+</sup>, asymptomatic individuals by Dr. Shearer's laboratory indicate that approximately 50% of these patients exhibit selective CD4<sup>+</sup> T helper cell functional defects without any AIDS symptoms and without loss of CD4<sup>+</sup> cell numbers (9267). A defect of antigen-presenting cell (APC) function was not observed in any such asymptomatic individuals. Many patients in various stages of disease exhibited a selective loss of CD4<sup>+</sup> T helper function but retained CD4<sup>-</sup> T helper function to HLA alloantigens. The results of this study: a) permit the detection of three stages of T helper dysfunction not previously defined by AIDS diagnostic tests; b) do not implicate



an APC defect early in disease progression; and c) provide evidence for an alternate (CD4<sup>-</sup>) T helper pathway that may be utilized for developing immunotherapy strategies in HIV<sup>+</sup> patients.

Studies by Drs. Laszlo and Dickler have identified a monoclonal antibody, DL-10, that detects a determinant (termed Ly-39.1) preferentially expressed on activated mouse B lymphocytes (9277). Expression peaks 60 hours after activation using LPS. The Ly-39.1 determinant is also expressed on splenic B lymphocytes and a subpopulation of bone marrow cells but at much lower levels and is absent from thymocytes, splenic T lymphocytes, and activated T lymphocytes. Mapping studies using BXD recombinant inbred mice indicate a gene controlling expression of the Ly-39.1 determinant is located on chromosome 17 between hbp-4 and acry-1. These results together with a unique strain distribution pattern suggest that Ly-39.1 is detecting a previously undescribed determinant selectively expressed on activated B lymphocytes which may be a receptor for lymphokines. Studies of B lymphocyte subpopulations indicate that irradiated large "activated" B lymphocytes, obtained directly from mice or B lymphoblasts induced *in vitro*, can augment responses of resting B cells stimulated with F(ab')<sub>2</sub> anti- $\mu$  and lymphokines. Proliferation was augmented 2-4 fold while antibody production was augmented 4-5 fold. This effect was specific for "activated" B lymphocytes in that other cell types did not have this effect. Kinetic experiments revealed that the augmenting signal was effective after stimulation via antigen receptors but prior to the effects of lymphokines. The augmenting effect does not appear to be genetically restricted. Investigation of the nature of the signal revealed that neither supernatants nor plasma membranes from activated B cells alone augmented responses but both together did. These studies suggest that interactions between B lymphocytes are important in regulating humoral immune responses.

## 2. CELL BIOLOGY OF IMMUNE RESPONSES

Dr. Stephen Shaw's laboratory has been identifying and characterizing the functions of cell surface molecules which facilitate T cell recognition ("accessory molecules"), particularly those which mediate adhesion (9257). ICAM-1, which they have previously characterized as a cell surface ligand for LFA-1, has been studied following immunoaffinity purification for its functional role not only in T cell adhesion but also as a costimulus for T cell activation. Its capacity to facilitate CD3-receptor mediated activation, together with other aspects of its regulation and function, suggest that it may be one of the most important "second signals" for T cell activation. Studies of other accessory molecules have identified CD44 as an additional member of the functionally important class of molecules involved both in T cell adhesion and activation. Studies of T cell/monocyte adhesion are characterizing the molecular basis of this immunologically important cell interaction.

Studies on the mechanism of lymphocyte-mediated cytotoxicity in Dr. Henkart's laboratory (9251) have shown that red blood cells are killed by both cloned cytotoxic T lymphocytes and helper T cells when linked to these cells by their T cell receptor. This cytotoxicity appears to operate by the same mechanism as the killing of more complex nucleated targets. Exocytosis of the granule cytolytic component of this lysis does not readily explain a substantial calcium-independent component of this lysis, nor does it account for the activity of cloned helper cells, which do not contain detectable levels of the cytolytic.

Other studies on the potent lytic agent cytolysin isolated from the secretory granules of cytotoxic lymphocytes have revealed that this protein can bind to target cells in a step prior to the lytic event. The lysis of the stable intermediate produced after this binding requires calcium. Studies on tumor target cells show that cytolysin can cause a sublethal membrane damage which can be repaired by the cells.

Dr. Henkart's laboratory (9263) has studied the role of secretory granule proteases in mediating target cell DNA breakdown during killing by cytotoxic T lymphocytes. By pretreatment of intact CTL with the covalent inhibitor PMSF granule proteases could be efficiently inactivated in the presence of agents which raise the pH of acidic intracellular compartments. This did not significantly inhibit the lytic efficiency of the CTL, but did greatly reduce target DNA breakdown. They have also found that cytotoxicity mediated by purified granules from CTL or NK tumors is accompanied by target DNA breakdown, and that this appears to require both cytolysin and another granule component. This component has been purified by assaying DNA release from detergent-isolated nuclei. In CTL granules this activity is identical to the previously described serine protease granzyme A. The mechanism by which this protease causes DNA breakdown is currently under investigation.

Studies in Dr. Alfred Singer's laboratory have identified both CD4<sup>+</sup> and CD8<sup>+</sup> lymphokine-secreting T-helper cells that initiated anti-MHC responses in vivo and in vitro (9256). Studies to identify any second signals involved in the activation of these two phenotypically distinct populations of T-helper cells revealed that IL-1 is a biologically active co-factor that together with ligand, either MHC class I or class II, will trigger either CD4<sup>+</sup> or CD8<sup>+</sup> Th cells to secrete IL-2. Dr. Alfred Singer's laboratory has also recently identified the first conventional antigen that will activate CD8<sup>+</sup> Th cells to secrete IL-2, namely vaccinia virus (9256). In these studies, Dr. Singer's laboratory found that CD8<sup>+</sup> Th cells constitute a portion of the Th cell population in virus infected mice, but that they are relatively short-lived and most prominent within several weeks of virus infection. These results are consistent with the hypothesis that CD8<sup>+</sup> IL-2 secreting Th cells require high ligand density for activation, a density generally only achieved by MHC alloantigens and replicating viruses.

Dr. Alfred Singer's laboratory has examined the molecular requirements for MHC recognition by class I allospecific T-killer cells (9256). These studies have identified a segment of the alpha-helical loop of the class I molecule to be a necessary docking site for most Kb-specific T cells. In other studies Dr. Singer's laboratory has found that multivalent cross-linking of the CD8 molecules expressed on mature T-killer cells actively induces them to differentiate into anti-CD8 resistant T cells (9256), suggesting that multivalent CD8 cross-linking induces an activation signal to mature CD8<sup>+</sup> T-killer cells.

Dr. Segal's laboratory has shown that antigen-presenting cells coated with certain types of bispecific antibodies present antigen to T cells at concentrations that would normally be too low to elicit a response (9254,9255). With normal mouse splenic B cells as a source of antigen-presenting cells, antigen targeted to sIgD or sIgM was more efficient in vitro than when targeted to class I or II MHC molecules, possibly because of the relatively rapid rate of

internalization following binding to sIg. Rapid internalization of antigen targeted with bispecific antibodies through its natural route, such as sIgD, was followed by storage and slow release of processed antigen to the B cell surface. By contrast, internalization through MHC molecules was slow, and was not followed by storage of antigen. However, the small amount of antigen internalized via surface MHC was efficiently processed and presented shortly after internalization. Further studies of antigen presentation *in vivo* have shown that the amount of antigen required for an antibody response in mice can be reduced  $10^3$ - $10^4$  fold, to nanogram levels, by targeting antigen to selected antigen presenting cells with bispecific antibodies instead of using antigen alone. However the enhanced response did not appear to involve B cells as antigen presenting cells in that bispecific antibodies containing anti class I or class II MHC but not anti-IgD antibodies were effective.

Dr. Hodes' laboratory has analyzed the mechanisms mediating the function of T helper cells in T dependent B cell antibody responses (9266). A role of both specific and non-specific T cell derived signals was demonstrated for activation of B cell antibody responses. It was shown that the supernatants of activated cloned T helper cells were capable of replacing these T cells in the antigen specific and MHC restricted induction of B cell IgG antibody responses. Two distinct and synergizing activities were identified in this supernatant: Interleukin 4, and an antigen-specific and MHC-restricted soluble factor. It was demonstrated that this latter factor can be affinity purified employing monoclonal antibodies specific for T cell receptor V $\beta$ 8 determinants. Biochemical analysis of  $^{35}$ S-methionine metabolically labeled cell-free material has shown that a disulfide-linked heterodimer indistinguishable from the cell surface TCR  $\alpha\beta$  heterodimer is released into the supernatant of multiple T cell lines studied. The  $\alpha\beta$  dimer in this material is associated with the CD3 $\epsilon$  chain and is not associated with other cell surface molecules such as MHC class I products. A mechanism for the rapid and directed secretion of mediators by T help was also demonstrated by analysis of granule exocytosis by T helper cells. Cloned helper cells, in response to T cell receptor mediated stimuli but not in response to IL2, rapidly secrete the contents of cytoplasmic granules. This provides a potential mechanism for the transmission of B cell activating stimuli from helper T cells to target B cells.

Studies by Drs. Laszlo and Dickler have shown that cross-linking of murine B lymphocyte Fc $\gamma$ RII receptors (FcR) by their specific ligand, together with ligand binding by B cell surface IgM, inhibits B lymphocyte differentiation at a particular stage in B-lymphocyte development (9253). Treatment of B cells with IL-4 or stimulation of B cells with F(ab') $_2$  anti-u or with a combination of PMA and CA $^{2+}$  ionophore results in diminished FcR ligand binding capacity, which consequently blocks FcR regulation of B lymphocytes. Inhibition of protein synthesis prohibits IL-4 and anti-u induced loss of complex binding, suggesting that this loss may be due to association of FcR with another membrane molecule.

### 3. TRANSPLANTATION BIOLOGY

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL), may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system developed in Dr. Gress' laboratory (9287), it was found that: 1) a cloned CTL population was sufficient to reject an allogeneic marrow graft; and 2) the mechanism by which these marrow



grafts were rejected was specific for MHC gene products expressed by the donor marrow and corresponded to the cytotoxic specificity of the clone. One approach to inhibit such graft rejection by CTL is by *in vivo* administration of monoclonal antibodies with specificity for CTL surface antigens. Administration of anti-CD3 to mice results in T cell activation within hours of administration, manifest by secretion of colony stimulating factors (CSF) detectable in the serum and produces extramedullary hematopoiesis in the spleen. In considering the use of anti-CD3 monoclonal antibody to enhance the engraftment of allogeneic marrow, these results indicate that it may be beneficial to time the injection of allogeneic marrow so that it is present during the period of T cell activation induced by the antibody in order to provide the possible benefit of factors which stimulate hematopoiesis.

Studies in murine marrow chimeras have suggested that MHC mismatched marrow transplantation is associated with immunoincompetence of the T cell arm of the immune response. To evaluate whether T cell maturation processes are comparable in mouse and man, the development of T cell responses in a primate, the rhesus monkey, has been studied in other experiments from Dr. Gress' laboratory (9288). The time course of CD4<sup>+</sup> and CD8/CD28<sup>+</sup> T cell reconstitution and the time course of the development of *in vivo* T cell immunocompetence (as defined by the ability to respond to an organ allograft) correlated with the number of T cells infused in autologous marrow, and did not correlate with marrow cell dose or with time of hematopoietic reconstitution, raising the possibility that residual T cells in the infused T cell depleted marrow played a central role in the generation of subsequent T cell populations. This would imply that the generation of T cells in marrow grafted primates may not be the same as in the mouse. Such differences would potentially have important implications for the transplantation of HLA-mismatched marrow in man.

Studies from Dr. Alfred Singer's laboratory have focused on the basic cellular mechanisms responsible for initiating and mediating transplantation responses *in vivo* (9275). Both isolated populations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were able, in isolation, to initiate and mediate skin allograft rejection responses, but did so by recognizing different classes of MHC alloantigens. Consequently, allograft rejection was not a function unique to one phenotypically distinct T cell subset. Indeed, Dr. Alfred Singer's laboratory found that *in vivo* skin allograft rejection responses required the initial activation of lymphokine secreting Th cells and the subsequent activation of lymphokine responsive Tk cells. These two T cell functions could either be mediated by interactions between distinct populations of Th and Tk cells, or, alternatively, could be mediated by a activation of a single population of dual function T cells that both secreted lymphokine and differentiated into cytolytic effector cells (9275).

In theory, *in vivo* allografts could either be destroyed by an antigen-non-specific lymphokine mechanism or by antigen-specific T-effector cells that assessed each individual cell in the graft for expression of alloantigens. To distinguish these mechanisms, Dr. Alfred Singer's laboratory constructed allophenic mice and assessed the antigen-specificity of the effector limb of the *in vivo* rejection response (9275). These studies revealed that the effector mechanism of *in vivo* allograft rejection is highly antigen-specific, destroying cells expressing alloantigens but sparing cells within the graft that do not.



Somewhat surprisingly, Dr. Singer's laboratory found a similar degree of antigen-specificity for Ia-restricted CD4<sup>+</sup> T-effector cells, even though most of the epidermal cells within a skin allograft are MHC class II negative (9275). It is likely from these findings that Ia<sup>-</sup> keratinocytes are induced to express Ia determinants during the in vivo rejection response, making them targets for Ia-restricted T-effector cells.

To ascertain that the immune mechanisms involved in rejection of skin allografts are also involved in rejection of other types of allografts, Dr. Alfred Singer's laboratory has examined the cellular basis for rejection of fetal pancreas allografts and isolated Islet cell allografts (9275). These studies revealed that the cellular basis for rejection of fetal pancreas allografts is identical to that involved in rejection of skin allografts (9275). In contrast, rejection of isolated Islet cell allografts is accomplished entirely by CD4<sup>+</sup> T cells, and not by CD8<sup>+</sup> T cells (9275). The basis for the limited rejection response against isolated Islet cell allografts is thought to be the absence of donor APC in the Islet cell allografts capable of activating CD8<sup>+</sup> Th cells (9275). Graft-versus-host disease (GvHD) is dependent in its generation on the presence of immune competent T cells in the donor marrow with specificity for allogeneic antigens of the host. One approach to preventing GvHD is the removal of these T cells from the marrow inoculum. Monoclonal antibodies with specificity for cell surface antigens expressed by human T cells have been used in Dr. Gress' laboratory (9271) to deplete normal T cells from allogeneic marrow and to remove malignant T cells from autologous marrow. A clinical protocol has been developed to assess the feasibility of utilizing these marrows in the treatment of aggressive T cell malignancies in combination with marrow ablative chemotherapy and radiotherapy.

Studies from Dr. Shearer's laboratory (9264) have focused on the effects on T lymphocyte function of treatment of mice with cyclosporin A (CsA). Exposure of mice in vivo with different doses of CsA indicate that lower doses of CsA selectively abrogate CD4<sup>+</sup> T helper function and activate T suppressor cells that selectively inactivate CD4<sup>+</sup> T helper function, whereas higher doses of CsA abolish both CD4<sup>+</sup> and CD8<sup>+</sup> T cell functions and activate non-T suppressor cells that inactivate all T cell functions tested. Preliminary studies in humans treated with different doses of CsA suggest similar patterns of T cell inactivation. These findings may be important for understanding the mechanism(s) responsible for the effects of CsA on allograft retention and may be useful in determining optimal doses of CsA for treatment of transplant patients.

Other experiments from Dr. Shearer's laboratory (9260) indicate that murine cytomegalovirus (MCMV) infection can synergize with a graft-versus-host (GVH) reaction to make the GVH more severe. MCMV was found to provide a class II-like signal to synergize with a class I GVH, but does not appear to provide a class I-like signal and synergize with a class II GVH. The antiviral drug DHPG can reduce MCMV infection, but does not reduce the severity of GVH in MCMV-GVH synergy. Studies of this type may help to elucidate the synergistic effects of CMV infection and GVH disease in human bone marrow transplantation.

#### 4. STRUCTURE, REGULATION AND FUNCTION OF GENES INVOLVED IN THE IMMUNE RESPONSE

Studies carried out in Dr. Dinah Singer's laboratory have demonstrated that the swine class I MHC multigene family contains only seven members, and as such is the smallest known class I family. Recent studies have been aimed at the characterization of the structure, expression, and function of this family.

Physical mapping has established that all of the genes are linked and resident within 500 kb. Determination of the DNA sequences of five members of the family have shown that the sequence organization of the class I SLA genes is similar to that of other class I genes in other species. Despite the small size of the family, three sub-groups of genes can be discerned based on their sequence homologies (9269). Analysis of class I cDNA clones has established the exon organization of the genes, as well as their expression. Only four class I genes appear to be expressed in vivo (9269, 9270). The three sub-groups defined by DNA sequence also display discrete patterns of expression. Thus, the genes of one sub-group are expressed ubiquitously, although their quantitative levels vary. A second sub-group defines a differentiation antigen. A third sub-group does not appear to be expressed at all (9270, 9276).

In order to investigate the molecular basis for the differential patterns of expression, Dr. Dinah Singer's laboratory has begun to generate and analyze transgenic mice containing isolated swine class I genes (9276). For one gene, encoding a classical transplantation antigen, it has been demonstrated that the pattern of expression of the swine gene in the transgenic parallels that observed in situ. Transgenics containing three other class I gene constructs have been generated.

Dr. Dinah Singer's laboratory has initiated studies to characterize DNA sequence elements and cognate trans acting factors associated with class I MHC genes (9270, 9276). A series of negative and positive regulatory DNA sequence elements within the 5' flanking region of the classical transplantation antigen gene have been identified. In particular a novel negative regulatory element has been described whose function is absolutely dependent on the presence of an enhancer element. This interaction is mediated by trans acting factors. Physical mapping of binding sites of trans acting factors has been achieved by both gel retardation and exonuclease protection assays. Although the molecular mechanisms by which the regulatory elements and their factor function is not known, the data suggest a model in which the negative and positive regulatory elements each define an independent binding site (9270).

Class I MHC gene expression is known to be induced by immunomodulators. Recent studies in Dr. Dinah Singer's laboratory have not only demonstrated the ability of a foreign class I gene in a transgenic mouse to respond to interferon, but have mapped the interferon responsive element. Furthermore, other studies in the same laboratory have demonstrated that ethanol, at physiologically attainable levels, is able to act as an immunomodulator by elevating steady state RNA and cell surface levels. Measurement of class I MHC antigen on peripheral blood lymphocytes in acutely intoxicated individuals showed a highly significant increase over controls (9270,9276,9210,9255)

## 5. TUMOR IMMUNOLOGY

Drs. Segal and Wunderlich have studied human blood lymphocytes from additional normal donors and confirmed that T lymphocytes can be targeted with bispecific antibodies in an MHC-independent fashion to block growth of established human ovarian cancer cells in an immunodeficient nude mouse model (9250,9254). In vitro tests show that multiple lineages of leukocytes can be activated and then directed to react against tumors by targeting with bispecific antibodies, including CD8<sup>+</sup>,CD56<sup>+</sup> T-cells, CD8<sup>+</sup>,CD56<sup>-</sup> T-cells, CD4<sup>+</sup>,CD56<sup>-</sup> T-cells, NK cells, and monocytes.

In the course of studying mechanisms for inducing MHC-independent antitumor cells, Dr. Wunderlich's laboratory has identified a detoxified form of lipopolysaccharide that synergizes with dextran sulfate to induce in vitro generation of murine cytotoxic antitumor cells (9250). The stimulatory action of these factors has been successfully adapted to in vivo induction of antitumor cells in the peritoneal cavity of mice. The response depends upon accessory cells, T-helper cells, and cytotoxic precursor cells, although the former two cell types can be bypassed with low doses of IL-2 in the stimulation mixture. The response results not only in activated killer cells that are directly cytotoxic for tumor cells but also in at least two populations of activated cells that can be targeted against tumors with bispecific antibodies: T cells targeted through CD3 determinants and NK/K cells targeted through FcR determinants.

## 6. FLOW CYTOMETRY

The Experimental Immunology Branch flow cytometry laboratory is managed by Susan Sharrow (9255). The laboratory currently supports over 80 individual research projects for more than 55 investigators. These investigations involve quantitative single cell analysis of parameters associated with cell types freshly prepared from different species/tissues, as well as spectrum of in vitro cultured cells. Basic research support is provided to members of the EIB and the Immunology Branch, as well as to other members of DCBD. Currently supported projects include a broad range of applications in the areas of cellular immunology, immunochemistry, transplantation biology, clinical immunology, immunogenetics, tumor immunology, and molecular immunology. These include, but are not limited to, the following studies: a) in vivo and in vitro analyses of intra-cellular signaling via T cell surface molecules, b) characterization of T cells from animals with genetic or induced immune dysfunction; c) studies of the pathogenesis of graft-versus-host disease; d) analyses of the correlations between T cell memory and the quantitative expression of cell surface adhesion molecules; e) investigations of T cell ontogeny and differentiation; f) studies of mechanisms of T cell repertoire generation; g) analyses of transplantation antigens and of gene regulation of expression of these antigens; h) investigations involving mechanisms and manipulations of antigen-presentation processes; and i) analyses of the mechanisms involved in marrow graft rejection versus acceptance (9256,9268,9273,9264,9259,9260,9276,9285,9254,9271,9287,9288,9255,9265).

Flow cytometric analysis has been a particularly critical element in Dr. Shaw's laboratory's systematic analysis of markers of human T cell heterogeneity (outlined above in Lymphocyte differentiation and regulation) (9257).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 09250-24 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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. Wunderlich Senior Investigator EIB, NCI

Others: D. Segal Senior Investigator EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6

## PROFESSIONAL:

1.5

## OTHER:

4.5

## CHECK APPROPRIATE BOX(ES)

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

B

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

We have confirmed by tests with peripheral blood from additional normal donors that human T lymphocytes can be successfully retargeted by heterocrosslinked antibodies to block the intraperitoneal growth of established human ovarian carcinoma cells in an immunodeficient athymic mouse model. Moreover, results of in vitro tests indicate that the retargeted antitumor cells represent less than 15% of the T cells and that inactive T cells become targetable if precultured with IL-2 and immobilized anti-CD3. In searching for a good in vitro correlate of in vivo antitumor activity, we have found that a 1-week in vitro tumor growth inhibition assay is a better indicator of retargeted antitumor activity in vivo than the 4-hour <sup>51</sup>Cr-release assay.

Two steps during the last year have led us closer to means for inducing AK activity in mice in a fashion less toxic than high-dose IL-2. First, AK antitumor cells have been induced in the peritoneal cavity of mice by the synergistic combination of IP injections of dextran sulfate and LPS, and oral indomethacin, which blocks prostaglandin induction by LPS. Second, a detoxified derivative of LPS has been identified that cooperates with polyanions to induce AK activity. Also, we have found that dextran sulfate induces generation of murine cytotoxic T cells can be retargeted with heteroconjugated mopoclonal antibodies. These advances offer new approaches to testing host antitumor activity in a mouse syngeneic tumor model.



## Project Description

Major Findings:

The focus of this laboratory has been on antitumor cytotoxic effector cells and factors that influence their generation. This year we have extended our studies of a) retargeting of human lymphocytes to human tumor cells, and b) MHC-nonrestricted cytotoxicity mediated by activated killer (AK) cells.

1. Retargeting the human immune system against human tumor cells. In collaboration with Dr. David Segal we are continuing our efforts to focus the immune system against selective tumors by redirecting the specificity of lymphocytes with heterocrosslinked antibodies. The heterocrosslinked antibodies are generated by chemically crosslinking monoclonal antibodies that react on one hand with triggering sites on the lymphocytes and on the other hand with antigens selectively expressed by tumor cells.

The major tumor model we use involves a human ovarian cancer cell line (OVCAR-3) that grows intraperitoneally in immunocompromised athymic mice. Growth of the tumor mimics important features of the human disease by generating massive ascites and invasive tumor implants, which progress to pulmonary metastasis and death by respiratory compromise and bowel obstruction.

Four to six days after mice are injected with tumor, we treat them over a 2-day period with four intraperitoneal injections of rIL-2 activated, retargeted human lymphocytes from a normal donor. At the time of treatment, mice have an established, growing tumor burden, and we estimate that the ratio of lymphocytes to tumor cells is 1:1 or less. Effects of the treatment on tumor growth are determined by measuring the amount of tumor in peritoneal wash fluid collected 15 days after tumor cells were initially injected. Without treatment the tumor burden increases about 30 fold over the 15 day period.

Continuation of last year's tests with additional donors has clearly established that treatment of tumor-bearing mice with retargeted human T lymphocytes significantly increases the incidence of tumor-free mice from heterocrosslinked antibody must link the T cells to tumor cells via triggering sites on the lymphocytes. Moreover, we have identified 3 different monoclonal antibodies that target T cells against OVCAR-3 cells when used in heterocrosslinks.

Additional cytotoxicity tests in vitro, using lymphocytes separated by buoyant density, showed that less than 15% of human peripheral blood T cells from each of 9 normal donors were effectively retargeted against tumor cells and that the active T cells were confined to relatively low density cells. For one donor whose cells were further tested, the targetable T cells were confined to the CD8<sup>+</sup>, CD56(NKH1)<sup>+</sup> phenotype. As a result of searching for means to effectively retarget more T cells, we have found that the high density T cells become targetable if preactivated by culture with IL-2 and immobilized anti-CD3. Activated, targetable T cells, originating from the high density population lose density with activation; however they may be of a different lineage than the original low density T cells in that they remain CD56 negative.

Finally, we have been searching for a better in vitro correlate of in vivo antitumor immune activity than found with  $^{51}\text{Cr}$ -release assays. The most promising test is a week-long tumor neutralization (Winn) assays in athymic mice and with 4-hour  $^{51}\text{Cr}$ -release assays using retargeted human T lymphocytes and a human colon adenocarcinoma line, LS174T. We have observed a correlation between results of in vitro tumor growth inhibition and in vivo assays in the following situations. Both CD8-depleted T-cells and high density T cells when retargeted show antitumor activity. Also, both the growth inhibition and the in vivo assay show innocent bystander antitumor effects when effector cells are retargeted to irradiated normal cells mixed with the tumor cells. The innocent bystander effect may be mediated by a cell-free cytotoxic factor that we have identified in the supernatant of the growth inhibition tests involving antitumor activity. By contrast, the  $^{51}\text{Cr}$ -release assay fails to detect antitumor activity by either retargeted CD8-depleted T cells or high density T cells. Moreover, innocent bystander effects have not been observed in the  $^{51}\text{Cr}$ -release assay.

2. MHC-nonrestricted cytotoxicity of murine activated killer (AK) cells against tumors. Previous work in this laboratory has shown that potent AK cell activity against tumors is generated primarily from NK cells, when mouse spleen cells are cultured for 5 days with small amounts of selected macromolecular polyanions and lipopolysaccharide (LPS), which act synergistically. Previous work has also shown that the response requires cell cooperation between accessory cells, T-helper cells and cytotoxic precursor cells and that the response does not simply involve enhanced production of IL-2 or interferon.

This year we have tested the feasibility of inducing AK activity in vivo with dextran sulfate and LPS, and we have also further explored the basis for the response that occurs in vitro. In the course of testing a variety of detoxified forms of LPS, we have identified a deacylated derivative of LPS, produced by Dr. Myers at Ribi Inc., that can replace native LPS as a cofactor for inducing AK activity. We have also used a combination of cofactors to induce AK activity in the peritoneal cavity of mice, namely oral indomethacin, which blocks LPS-induced production of prostaglandins, and IP injections of LPS and dextran sulfate. In vitro, LPS and dextran sulfate induce not only AK cells, but also targetable cytotoxic T cells, as shown by retargeting tests in vitro with heterocrosslinked antibodies that act on T cells. We anticipate that dextran sulfate, indomethacin, and detoxified LPS can be used in vivo with heterocrosslinked antibodies to provide protection against tumor growth in a syngeneic mouse tumor model.

The mechanism of action of LPS appears to involve the release of  $\text{TNF}\alpha$  and/or interferon  $\beta$  in that these cytokines synergize with dextran sulfate, but not with LPS, to induce generation of AK cells. LPS is known to stimulate release of both cytokines,  $\text{TNF}\alpha$  arising primarily from macrophages and interferon  $\beta$  from T helper cells. A fundamental role for Ia expression by accessory cells is clear from the observations that anti-Ia antibodies block inductions of AK cells by dextran sulfate and LPS and that L-cells transfected with Ia genes syngeneic but not allogeneic to the responding lymphocytes induce high levels of AK activity by splenocytes even in the absence of dextran sulfate and LPS.

Proposed Course of Project:

1. Retargeting the immune system against human tumor cells. In collaboration with Dr. David Segal we plan to pursue the following issues involving retargeting human lymphocytes against human ovarian cancer cells in the athymic mouse model: 1) study the effects of retargeting on host survival, 2) retarget leukocytes other than T-cells, such as NK cells, neutrophils and monocytes, by using in the heterocrosslinked antibody preparations of monoclonal antibodies against Fc receptors, 3) determine the effects of retargeting against larger in vivo tumor burdens, 4) determine whether retargeted cells injected IV will come to tumor in the peritoneal cavity, and 5) initiate an all-mouse model for testing the effects of retargeting in vivo against tumors.

2. MHC-nonrestricted cytotoxicity of activated killer (AK) cells against tumors. We plan to investigate the following issues concerning generation of AK cells. 1) Will induction of AK cell and T cell activity IP in mice by methods described above, combined with retargeting antibodies, promote in vivo tumor rejection? 2) Roles for cytokines (eg TNF $\alpha$  and INF $\beta$ ), which alone are insufficient, have been identified in the AK response induced by polyanions and LPS. Can additional relevant cytokine intermediates be detected by testing the supernatants of cultured accessory cells pulsed with LPS, by using anticytokine neutralizing antibodies, and by addback tests with purified cytokines? 3) Will the same rules that apply to generating AK cells with polyanions and LPS also apply to generation of veto activity, which we have observed among thymocytes stimulated in vitro by IL-2.

Publications:

Segal DM, Garrido MA, Perez P, Titus JA, Winkler DA, Ring DB, Kaubisch A, Wunderlich JR. Targeted cytotoxic cells as a novel form of cancer immunotherapy. Mol Immunol 1988;25:1099-1103.

Segal DM, Snider DP, Perez P, Qian J-H, Titus JA, Garrido MA, Winkler DA, Wunderlich JR. The use of heterocrosslinked monoclonal antibodies in targeting cytotoxic cells and in facilitating antigen presentation. Acuta Sandoz 1989;14:58-70.

Wunderlich JR, Shearer GM. The <sup>51</sup>Cr-release assay for lysis of target cells by cytotoxic T-lymphocytes (CTL). In: Coligan JE et al Ed. Current protocols in immunology. 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09251-19 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Target Cell Damage by Immune Mechanisms

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

PI:	P.A. Henkart	Senior Investigator	EIB, NCI
Others:	K. Andrews	Microbiologist	EIB, NCI
	M. Kubicek	Microbiologist	EIB, NCI
	A. Kuta	Guest Researcher	EIB, NCI
	J. Shiver	Guest Researcher	EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

2.5

## OTHER:

0.5

## CHECK APPROPRIATE BOXES)

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

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Both cytotoxic T lymphocytes and cloned helper T lymphocytes have the ability to cause rapid lysis of mammalian red blood cells in the presence of crosslinked hybrid antibodies against the T cell receptor and a target membrane antigen. Studies of the physiology of CTL lysis of the red cells showed that, in contrast to lysis of nucleated cells, no measurable killer cell independent phase of the lytic process was detectable. The helper T cells did not contain measurable levels of granule cytolysin as shown by rbc lysis of purified granules, by immunofluorescence using anti-cytolysin antibodies, or by Northern blots. Substantial levels of cytotoxicity remained in the presence of EGTA, suggesting that the granule exocytosis mechanism cannot explain at least some of the lytic activity.

The mechanism of lysis by LGL granule cytolysin as been analyzed by identifying an intermediate in the lytic process which is stable in both low pH and low ionic strength (in isotonic sucrose). The cytolysin in this intermediate was found to be displaced from the cell by treatment with 1N NaCl, indicating that it was not inserted into the membrane.



## Project Description

Major Findings:

In this project we are pursuing three different approaches to understanding the mechanism of lymphocyte mediated cytotoxicity and particularly the validity of the granule exocytosis model previously described in our laboratory.

In the first approach, the mechanism of lysis by LGL granule cytolysin has been analyzed by taking advantage of the observation that low pH and low ionic strength (in isotonic sucrose) block lysis at a step after cytolysin binding to red cells. Thus we can wash off all unbound cytolysin and produce a stable intermediate which will hemolyse when resuspended in a buffer containing isotonic saline at neutral pH. Calcium is required for this lytic step by either route of intermediate formation. We have found that incubation of either intermediate, using either red cells or tumor cells, with 1N NaCl causes cytolysin to be released in active form from the cell into the medium. This indicates that no insertion into the membrane lipid bilayer has occurred. We also find that a non-cytolysin granule component stabilizes the intermediates which are formed. This appears to be the granule proteoglycan, based on its resistance to preteolytic digestion, and its sensitivity to chondroitinase.

In another approach, we have studied the killing of mammalian red blood cells as simple target cells for CTL since these cells are unlikely to undergo a postulated "programmed cell death" or to have receptors for cytotoxic lymphokines; we thus predicted that they would utilize the granule exocytosis pathway of cytotoxicity. Using heteroconjugates, i.e., crosslinked hybrid antibodies against the T cell receptor and a red cell membrane antigen, efficient lysis of both red cell and nucleated targets could be achieved using cloned CTL or helper T cells. In most experiments removal of calcium from the medium using the chelator EGTA left a significant level of killing; under these conditions, neither granule exocytosis nor cytolysin activity. The T helper clones do not contain detectable cytolysin in their granules, nor is cytolysin detectable by indirect immunofluorescence of detergent permeabilized fixed cells. These results suggest that another pathway and lytic agent is responsible for the cytotoxicity.

Proposed course:

The ability of T lymphocytes to kill red blood cells by a non-granule exocytosis pathway is a critical issue for the lymphocyte cytotoxic mechanism field. We are currently testing the T helper clones which kill for the presence of mRNA specific for cytolysin/perforin. If this is negative, the evidence will be strong that an alternative pathway exists, and further work on the physiology of this cytotoxicity will be undertaken with an aim of understanding it on a molecular basis.

The principle approach we are taking to test the applicability of the granule exocytosis model is by transfecting the rat basophilic leukemia cell line (RBL) with the gene for cytolysin (perforin). The RBL cells were chosen since they have a IgE-triggered regulated secretory pathway utilizing acidic granules containing lysosomal enzymes. They do not normally express cytolysin nor do they kill, but when transfected with the appropriate gene we predict that they should be capable of lysing IgE coated target cells by the granule exocytosis pathway. At the present stage we have taken the mouse cDNA clone gene, trimmed away most of the 5' upstream region and inserted the gene into the pSVL expression vector (which uses a SV40 promoter) in the correct orientation.

Publications:

Bashford CL, Menestrina G, Henkart PA and Pasternak CA. Cell damage by cytolysin: Spontaneous recovery and reversible inhibition by divalent cations. J. Immunol. 1988;141:3965-3974.

Kuta AE, Reynolds CR and Henkart PA. Mechanism of lysis by LGL granule cytolysin: Generation of a stable cytolysin-RBC intermediate. J. Immunol. 1989; in press.

Kolber MA, Quinones RR, and Henkart PA. Target cell lysis by cytotoxic T lymphocytes redirected by antibody-coated polystyrene beads. J. Immunol. 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09252-17 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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 Senior Investigator EIB, NCI

Others: S. A. Rosenberg Chief SB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## 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

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
Recurrence	32	25	38	25
Deaths	24	23	27	21

In vitro assays have only been carried to the point of indicating that patients are being effectively immunized with the vaccine (see Project No. Z01-CB-05016-10 I). In addition, PPD tests indicate that all patients receiving BCG have converted to a positive skin test.

Proposed Course of Project:

No further patient accrual will occur. All treatment has been completed and, for those patients already on study, follow-up as described by the IB-2 protocol will be continued.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09253-17 E

## PERIOD COVERED

October 1, 1989 to March 31, 1989

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

Function of B Lymphocyte Fc $\gamma$  Receptors

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

PI: G. Laszlo Visiting Fellow EIB, NCI  
 Others: H. B. Dickler Chief CIB, NIAID

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

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 function of B lymphocyte Fc $\gamma$  receptors. Previous findings indicate that the Fc $\gamma$  receptors of B lymphocytes interact with: a) the lymphocyte cytoskeleton, b) Ia antigens, c) LyM antigens, d) surface IgM, and e) surface IgD. Each of these interactions is distinct, specific, and non-random. Studies utilizing antigen-antibody complexes indicate that B lymphocyte Fc $\gamma$  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 lymphocytes surface IgM by a separate ligand is necessary for inhibition to occur, suggesting that the previously described interaction between these two membrane receptors may be involved in generating the negative signal. Studies using monoclonal anti-Fc $\gamma$  antibody (2.4G2) in a variety of forms including native, chemically cross-linked into homodimers or heterodimers with anti- $\delta$  or F(ab') $_2$  anti- $\mu$  antibodies, and on a Sepharose matrix indicate that the monoclonal antibody only generates the negative regulatory signal if effective cross-linking of the receptor is obtained. IL-4 specifically induces a marked (90%) loss of binding of complexes to B lymphocyte Fc $\gamma$ R II. The IL-4 induced loss of binding of complexes was time and temperature dependent and was reversible. IFN- $\gamma$  could partially prevent this effect. The loss of binding of complexes did not appear to be due simply to a loss of expression of Fc $\gamma$ R II because IL-4 induced only a moderate reduction of two Fc $\gamma$ R II specific monoclonal antibodies. Stimulation of B lymphocytes with F(ab') $_2$  anti- $\mu$  or with a combination of PMA and CA $^{2+}$  ionophore induced a similar loss of binding of complexes, suggesting that loss of Fc $\gamma$ R II ligand binding capacity can occur via 2 pathways. Inhibition of protein synthesis prohibits IL-4 and anti- $\mu$  induced loss of complex binding, suggesting that this loss may be due to association of Fc $\gamma$ R II with another membrane molecule.

## Project Description

Major Findings:

Experiments with antigen-antibody complexes (IgG anti-ovalbumin-ovalbumin or IgG anti-IgG-IgG) have revealed that such complexes inhibit the antibody secretion (60-70%) of normal B cells responding to F(ab')<sub>2</sub> anti-mu plus lymphokines. Complexes in antigen excess were most effective. Maximal inhibition was seen with as little as 0.2-0.5 ug/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-course experiments indicated that inhibition was real and not simply a change in the kinetics of the response. Complexes were only effective if added to culture 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 down regulatory signal. Thus, the downregulatory signal is generated cooperatively by Fcγ receptors (surface IgM). The previously described interaction between these two receptors may play a role in the generation of the downregulatory signal.

Studies with monoclonal anti-Fcγ receptor antibodies (2.4G2) indicated that in native form they did not affect B lymphocyte responses. This was true over a broad dose range and when give simultaneously or 24 hours prior to B lymphocyte stimulation. Moreover, homodimers made with 2 different chemical crosslinkers also did not affect responses. Binding studies indicated that such preparations were cross-linking at least 4 FcγR. Attempts to produce a large homomultimers resulted in inactivation of the 2.4G2. In contrast, 2.4G2 chemically cross-linked into heterodimers with F(ab')<sub>2</sub> anti-mu, or bound to a Sepharose matrix inhibited B lymphocyte responses in a fashion similar to antigen-antibody complexes. These studies indicate that monoclonal anti-FcγR antibodies only downregulate B lymphocyte responses if sufficient cross-linking of the receptor is obtained, and that internalization of the ligand is not required. These studies suggest that 2.4G2 can be used to regulate B lymphocyte responses if utilized in an appropriate form.

B lymphocytes from various sources have been assessed for their susceptibility to Fc $\gamma$  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 Fc $\gamma$  receptor mediated downregulation may play a role in the hyperresponsiveness of NZB B lymphocytes.

B lymphocytes cultured for 24 h with recombinant IL-4 lost (90%) the capacity to bind IgG complexes as assessed by flow microfluorometry. Specificity was indicated by the observations that: IL-1, IL-2, IL-3, GM-CSF, and IFN- $\gamma$  did not inhibit the binding of complexes; IL-4 did not have a similar effect on other B lymphocyte membrane molecules; and inhibition was completely prevented by monoclonal anti-IL-4 (11B11). Inhibition was dose-dependent (>60% inhibition with 1 U/ml and maximal with  $\geq 30$  U/ml), time dependent (no effect at 4 h and maximal at 24 h), temperature dependent (no effect at 4°C), and reversible (cells which had lost the capacity to bind complexes due to IL-4 regained this ability when recultured for 24 h in the absence of IL-4). The inability to bind complexes could not be accounted for solely by a loss of expression of Fc $\gamma$ R II because IL-4 induced only a moderate reduction in the binding of two Fc $\gamma$ R II specific monoclonal antibodies (20% for 2.4G2 and 32% for K9.361). These results suggest that IL-4 regulates the binding of complexes to B lymphocyte Fc $\gamma$ R by inducing some form of alteration of the receptor. This effect could play a role in the regulation of B lymphocyte responses by preventing Fc $\gamma$ R II mediated downregulation.

Murine B lymphocytes cultured with F(ab')<sub>2</sub> anti-mouse  $\mu$  or  $\delta$  lost (85%) the capacity to bind antigen-IgG antibody complexes as assessed by flow microfluorometry. Similar to the previously described IL-4 induced loss of Fc $\gamma$ R II ligand binding capacity, anti- $\mu$  induced loss of binding of complexes was concentration, time, and temperature dependent, reversible, and not due to decreased expression of the receptor because binding of monoclonal anti-Fc $\gamma$ R II to B lymphocytes cultured with anti- $\mu$  was unaffected. Activation of PKC and elevation of [Ca<sup>2+</sup>]<sub>i</sub> may be involved in the anti- $\mu$  induced loss of binding of complexes because culturing B lymphocytes with the combination of PMA and Ca<sup>2+</sup> ionophore induced a similar loss of binding of Cx. Because IL-4 does not activate PKC or elevate [Ca<sup>2+</sup>]<sub>i</sub> this result also suggests that IL-4 and anti- $\mu$  may alter Fc $\gamma$ R II ligand binding capacity via different pathways. Inhibition of protein synthesis largely prevented IL-4 and anti- $\mu$  induced loss of binding of complexes to Fc $\gamma$ R II, suggesting that this loss may be due to association of the receptor with another membrane molecule. In contrast to the effects of other activators, LPS caused increased expression of B lymphocyte Fc $\gamma$ R II (binding of both complexes and monoclonal anti-Fc $\gamma$ R II increased three fold). Thus, B lymphocyte activators alter Fc $\gamma$ R II expression or ligand binding capacity and can thereby affect Fc $\gamma$ R II generated regulatory signals.

Publications:

Dicker HB, Kubicek MT. Effects of various form of monoclonal anti-Fc $\gamma$ R II (2.4G2) on B lymphocyte responses. Mol Immunol 1988; 25:1169-1174.

Laszlo G, Dickler HB. Interleukin 4 induces loss of B lymphocyte Fc $\gamma$ R II ligand binding capacity. J Immunol 1988;141:3416-3421.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09254-15 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

The Manipulation of Immune Processes with Heterocrosslinked Antibodies

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

PI: D. M. Segal Senior Investigator EIB, NCI

Others: D. P. Snider Visiting Fellow EIB, NCI  
 J-H. Qian Visiting Fellow EIB, NCI  
 D. Mezzanica Guest Researcher EIB, NCI  
 S. Andrew Visiting Fellow EIB, NCI  
 J. Wunderlich Senior Investigator EIB, NCI  
 M. Garrido Visiting Fellow EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

8.0

## PROFESSIONAL:

6.5

## OTHER:

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.)

1. Cytotoxicity and tumoricidal activities targeted with bispecific antibodies have been studied in PBL with respect to the types of cells mediating these activities and their activation requirements.
2. Targeted cytotoxic cells from human peripheral blood have been used to eradicate intraperitoneal growth of human ovarian carcinoma in nude mouse models.
3. Heterocrosslinked antibodies have been used to enhance antigen presentation and to study the mechanisms of antigen presentation. In vivo studies show that such antibodies can enhance the efficiency of immunization.

## Project Description

Major Findings:*Targeted Cytotoxicity: in vitro studies*

We have previously shown that cytotoxic cells can be induced to lyse cells other than their natural targets with antibody heteroconjugates which contain an antibody against the cytotoxic cell receptor crosslinked to an antibody against a target cell structure, for example a tumor antigen. We are continuing our studies on identifying the activities within human peripheral blood lymphocytes (PBL) that are targetable with heteroconjugates, and studies on ways of generating increased activities for potential therapeutic purposes. Studies carried out during the past year have shown that targeted cytotoxic cells in human PBL fall into two major categories; low and high buoyant density cells that can be separated on discontinuous Percoll density gradients. The low buoyant density fraction contained all of the targetable T cell (cytotoxicity dependent upon anti-CD3 containing heteroconjugates) and K cells (cytotoxicity dependent upon anti-Fc $\gamma$ RIII containing heteroconjugates) activities in fresh PBL. Both types of low density cells require IL-2 for maintenance of cytotoxic activity and mediate MHC unrestricted lysis. All of the cytotoxic activity in the low density T and K cells resides in the CD56 (NKH1)<sup>+</sup> subset. CD57 (HNK1) divides the CD56<sup>+</sup> cells, with targetable T and K cell activity residing in both CD57<sup>+</sup> and CD57<sup>-</sup> cells. Our studies have shown that the cells in fresh PBL mediating targeted T cell cytotoxicity are CD56<sup>+</sup>, CD3<sup>+</sup>, and CD8<sup>+</sup>; this subset typically represents about 2% of PBL. Fresh PBL, or PBL incubated for short periods with rIL-2 are reasonably potent effector cells in the presence of anti-CD3 containing heteroconjugates. The 2% of cells responsible for this activity must therefore be very potent mediators of cytotoxicity.

The high buoyant density lymphocytes obtained from Percoll gradients, have no targetable activity, but are able to generate over several days, targeted T cell activity in the presence of a T cell receptor crosslinking signal (immobilized anti-CD3, lectin, or OKT3 in the presence of monocytes) plus lymphokines. The activated high buoyant density effector T cells differ from the low density cells in that they do not express CD56 or CD57. Moreover, both CD4<sup>+</sup> and CD8<sup>+</sup> activated high density T cells are able to mediate targeted lysis, whereas only CD8<sup>+</sup> low density cells T cells mediate lysis. In addition, low density cells mediate MHC unrestricted lysis in the absence of antibody, whereas the activated high density cells do not. These data suggest that there two distinct classes of cytotoxic T cell and one class of K cell in human peripheral blood. All three of these activities should be taken into account when designing in vivo therapeutic uses for targeted cytotoxic cells (see below).

*Targeted Tumor Neutralization*

Our first in vivo studies employed classic Winn-type tumor neutralization assays, using the LS174T human colon adenocarcinoma line, which forms tumors when injected subcutaneously in nude mice. In these studies targeted T and K cells were both able to prevent the establishment and growth of the tumors at low effector:target ratios (eg at 1:1 or less). Because all of the targeted T cell cytotoxicity as measured in a <sup>51</sup>Cr-release assay resided in the CD8<sup>+</sup>, CD4<sup>-</sup> subset, we expected the tumor neutralizing activity to be in this subset as well. However we found that most of the tumor neutralizing activity was in the

CD4<sup>+</sup> subset, with some activity remaining in the CD8<sup>+</sup> cells. It was clear from these studies that tumor neutralization and <sup>51</sup>Cr release measured two different activities.

Because CD4<sup>+</sup> PBL were unable to lyse LS174T human colon adenocarcinoma cells but were able to block subcutaneous tumor growth in nude mice in the presence of a hetero-conjugate containing anti-CD3 crosslinked to an anti-tumor antibody (anti-CD3 x anti-tumor), we devised an in vitro tumor growth inhibition assay in order to better reflect the in vivo situation. Experiment were done in which LS174T cells were incubated for various times with PBL in the presence or absence of antibody hetero-conjugates. In the absence of PBL or in the presence of PBL without heteroconjugate, the tumor cells grew progressively, reaching a plateau at about day 9. In the presence of PBL plus anti-CD3 x anti-tumor, the tumor growth was markedly inhibited, while tumor growth was slightly delayed by the PBL from this donor in the presence of anti-CD16 x anti-tumor. The anti-tumor effect was dependent upon effector:target ratios, typically titrating out at a PBL:tumor ratio between 3:1 and 10:1. Tumor growth inhibition was also dependent upon the presence of crosslinked antibody. In the presence of PBL, either antibody alone, or an uncrosslinked mixture of the two antibodies used to make a heteroconjugate did not result in the blockage of tumor growth.

Next we tested whether CD8 depleted PBL could mediate lysis and tumor growth inhibition. We found that depletion of the CD8<sup>+</sup> cells removed most of the targeted cytotoxic activity but not the ability to block tumor growth. Because we had found that all targetable cytotoxic activity was in a low buoyant density fraction of PBL, we separated high and low density PBL on a Percoll density gradient and tested them for the ability to mediate lysis and tumor growth inhibition. As expected from our previous results, essentially all of the cytotoxic activity of targeted T and K cells, in addition to the NK like activity in the absence of antibody was mediated by the low buoyant density cells. By contrast, the high and low density cells were equally active in blocking tumor growth in the presence of anti-CD3 x anti-tumor. The low, but not the high density cells were able to block tumor growth in the presence of anti-CD16 x anti-tumor. The high density cells contain very few if any CD16<sup>+</sup> cells, so it is not surprising that the anti-CD16 containing heteroconjugate did not induce these cells to block tumor growth.

Given that the high buoyant density T cells could block tumor growth but would not mediate lysis in the presence of anti-CD3 x anti-tumor, we asked whether the same relation would hold in a Winn type tumor neutralization assay. Nude mice were given subcutaneous injections of LS174T cells in the presence of low or high buoyant density PBL, with or without anti-CD3 x anti-tumor. We found that the high density cells were able to mediate tumor neutralization in this assay, in a heteroconjugate-dependent fashion. The low density fraction was even more efficient at blocking tumor growth in a Winn type assay. Thus, our experiments clearly show that cells that do not mediate lysis in a <sup>51</sup>Cr release assay are competent to block tumor growth in vitro and in vivo.

It has been established in several systems that bystander cells are not lysed in 4 hr <sup>51</sup>Cr release assays of targeted cytotoxicity. Our experiments show that in this regard, tumor growth inhibition is fundamentally different from cytotoxicity. We performed experiments in which PBL were incubated with tumor cells in the presence of either anti-CD3 (Fab) x anti-tumor (Fab), or anti-CD3 (Fab) x anti-K<sup>k</sup> (Fab). As expected, tumor growth was inhibited when anti-CD3



(Fab) x anti-tumor (Fab) was added, but was unaffected by anti-CD3 (Fab) x anti-K<sup>k</sup> (Fab), since the tumor used in these studies did not express K<sup>k</sup>. However, the addition of C3H (H-2<sup>k</sup>) thymocytes to cultures containing PBL, tumor cells, and anti-CD3 (Fab) x anti-K<sup>k</sup> (Fab) resulted in the inhibition of growth of the bystander tumor cells.

We next tested whether targeted T cells could block the growth of bystander cells *in vivo*, in Winn type tumor neutralization assays. We found that in the presence of anti-CD3 (Fab) x anti-K<sup>k</sup> (Fab), the growth of tumor cells was blocked when H-2<sup>k</sup>, but not H-2<sup>b</sup> spleen cells were injected with tumor cells, PBL, and heteroconjugate subcutaneously into immunocompromised mice. Our experiments therefore showed that targeted human T cells can block the growth of bystander tumor cells *in vivo*.

Because tumor growth inhibition did not require a directly linkage between PBL and tumor cells, experiments were done to determine whether anti-CD3 alone would induce PBL to block tumor growth. Several monoclonal antibodies against cell surface components on PBL were adsorbed to the bottoms of plastic tissue culture flasks, and PBL and LS174T tumor cells were added. Anti-CD3 x Anti-tumor was used as a control. We found that adsorbed anti-CD3 was as effective at blocking tumor growth as the heteroconjugate. Adsorbed anti-CD4, CD8, or CD16 were unable to reproducibly elicit a strong anti-tumor response. When anti-CD3 was added in solution to tumor cells plus PBL, tumor growth was not inhibited unless monocytes were present, and anti-CD3 F(ab) did not induce inhibition of tumor growth in the presence of monocytes. This suggests that the tumor growth inhibitory activity is elicited from peripheral blood T cells when TcR are crosslinked, either through heteroconjugate plus target cells, anti-CD3 adsorbed to plastic, or anti-CD3 crosslinked by Fc<sub>γ</sub>R on monocytes.

The simplest explanation for the observed bystander effects is that TcR crosslinking induces peripheral T cells to secrete factors that block tumor growth. In order to test this hypothesis, PBL were cultured in flasks coated with either anti-CD3 or with no antibody. At various times, supernatants were removed, centrifuged and tested for the ability to block LS174T growth. These experiments showed that supernatants of PBL grown on CD3-coated flasks were able to block tumor growth at greater than 54 fold dilutions, whereas supernatants from cells grown in the absence of antibody exhibited a very weak anti-tumor effect. The maximum amount of growth inhibition factor was obtained at day 3 of incubation, the activity decreasing thereafter, suggesting that the factor is not stable in culture. We have observed large donor variabilities in the amount of factor secreted. The factor was produced by both low and high buoyant density cells. Low density cells usually produced greater amounts of the factor than the high density cells, and often produced significant amounts in the absence of receptor crosslinking. We have tested the factor against a number of tumor lines for the ability to block growth. In addition to LS174T, the growth of OVCAR3 (human ovarian carcinoma), B16 (murine melanoma), and K562 (human myelogenous leukemia) was blocked by the factor. RBL5 (murine leukemia) and F2B (human leukemia) were refractory to inhibition by the supernatants.

#### *Targeted Cytotoxicity: in vivo studies*

We have established a system to test whether targeted cytotoxic cells could eradicate an established tumor. In these studies we have treated intraperitoneal growth of the human ovarian adenocarcinoma line, OVCAR3, with



intraperitoneal injections of targeted PBL. We chose this system because both the tumor and the treatment are confined to the peritoneum, thereby minimizing problems of delivering the targeted cytotoxic cells to the tumor. Since OVCAR3 grows ip in nude mice in much the same way that ovarian carcinoma grows in female cancer patients, these studies could form the basis for initiating clinical trials, using targeted PBL to treat ovarian cancer. Nude mice are given tumor cells ( $5 \times 10^6$  -  $1 \times 10^7$  per mouse) on day 0 and are treated with four doses of PBL alone or PBL plus heteroconjugate on days 5 and 6,  $10^7$  cells per dose at 12 hr intervals. They are then sacrificed on day 15 and tested for tumor growth by ip lavage. Positive mice give a large tumor cell pellet upon centrifugation of the peritoneal wash, while negative mice have no detectable tumor cells.

Since last year, we have obtained statistically large samples that clearly show that PBL, incubated overnight with IL-2, can, in the presence of some heterocrosslinked antibodies, cause established tumor to disappear. The results are statistically significant to P values less than  $10^{-5}$ , and we have shown that three different anti-tumor antibodies linked to anti-CD3 will mediate this effect. More recently we have been testing PBL that have been activated by immobilized anti-CD3 and IL-2, in this assay. This treatment produces large amounts of cytotoxic activity from the high buoyant density fraction, and early results suggest that this treatment is even more effective at eradicating tumor growth than using cells that are only briefly stimulated with IL-2.

#### *Targeted Antigen Presentation*

T helper cells recognize antigen in a degraded form, bound to MHC class II molecules on the surfaces of APC. Non-specific APC take up antigen by fluid phase pinocytosis, a relatively inefficient process requiring high antigen concentrations. The pinocytosed antigen is then degraded and returned to the surface in association with class II molecules. We have shown that non-specific APC can be rendered specific by targeting antigen to their surfaces with heterocrosslinked antibodies. Thus heteroconjugate-coated APC can present antigen to T cells at concentrations which would normally be too low to elicit a response. The presentation of antigen to T cells can lead to the expansion and activation of antigen-specific T-helper clones, which in turn are essential for the generation of cellular and humoral responses against that antigen.

We used heteroconjugated antibodies to bind hen egg lysozyme (HEL) to various structures on the surface of normal splenic B cells, to determine which structures would provide the best targets for enhanced presentation. We found that HEL was presented efficiently to hybridoma T cells if bound to sIgD, sIgM, or class I or II MHC molecules, but not at all if bound to Fc $\gamma$ RII, or B220 molecules on B cells. The efficiency of presentation of HEL was measured as a function of the amount of  $^{125}$ I-HEL bound per cell. HEL was presented with 5-10 times greater efficiency when bound to sIg, than when bound to MHC molecules. When compared on the basis of the amount of HEL bound, sIgD and sIgM functioned equally as target structures, as did class I and class II MHC molecules. Large amounts of HEL bound to B220, but no presentation resulted, indicating that focusing HEL to the APC surface was not sufficient for presentation to occur. HEL was internalized rapidly and in large amounts when bound to sIgD or sIgM, but slowly and in small amounts, when bound to class I or class II MHC molecules. Thus, a rapid rate of internalization may in part explain the high efficiency of antigen presentation after binding to sIg. However, the small

amount of HEL internalized via MHC molecules was utilized efficiently for presentation. These results indicate that sIgM and sIgD serve equally on normal B cells to focus and internalize antigen and enhance antigen presentation, but that class I or class II MHC molecules can also be used to internalize antigen and enhance antigen presentation, perhaps by a separate intracellular processing pathway.

We have also studied in detail, the internalization, processing and presentation of HEL bound to surface IgD or MHC-encoded molecules on normal murine B cells, using bispecific heteroconjugate antibodies. We found that nearly all HEL bound to IgD was internalized rapidly, and was stored for up to 20 hours, with at least a portion in chloroquine-sensitive, low pH compartment(s) at all times. Degradation and presentation of HEL to hybridoma T cells began several hours after internalization via IgD, but intact HEL was still present in the cells by 20 hours of culture. In contrast, very little HEL was internalized and stored, if bound to class I or class II MHC molecules, and both presentation and degradation of HEL was slower than with HEL bound to IgD. However, the small amount of HEL internalized via surface MHC, was efficiently processing and presented. These experiments show that internalization of antigen through its natural route (ie through sIgD) on B cells is followed by the storage and slow release of processed antigen to the B cell surface. By contrast, internalization through MHC molecules is slow, and is not followed by storage of antigen. Antigen internalized through MHC molecules appears in a form recognizable by T helper cells, shortly after internalization.

Finally, we have been doing experiments to show that antigen targeted to APC in vivo would be more immunogenic than untargeted antigen. Since the antibody response of mice to HEL is T cell dependent, we immunized mice with HEL and bispecific crosslinked antibodies, in an attempt to target HEL to various APC in vivo and enhance the HEL-specific IgG antibody response. The amount of HEL required for an antibody response in mice was reduced  $10^3$ - $10^4$  fold (to nanogram amounts) using crosslinked antibody specific for class II MHC molecules. HEL mixed with the crosslinked antibody was as immunogenic as HEL emulsified in incomplete Freund's adjuvant. Crosslinked antibodies specific for class I MHC, or for Fc $\gamma$ RII also targeted HEL efficiently, but crosslinked antibodies specific for IgD did not.

### Proposed Course of Project

#### 1. *Ovarian cancer project.*

Our current goal is to optimize conditions for eradicating ip ovarian tumor in nude mice. There are several aspects of this problem which we will investigate. (1) We will test heteroconjugates containing several different anti-tumor antibodies to find those which work best. (2) We will try to find better ways of activating PBL in order to get increased anti-tumor activities from all donors. (3) We will compare anti-tumor activities of targeted T cells with those of the K cells in the ovarian model, and then test whether monocytes or neutrophils, targeted with either anti-Fc $\gamma$ RI or II might also be able to eradicate established tumors. (4) We will vary the treatment schedules, with a special interest in determining how long treatment can be delayed without loss of anti-tumor activity. (5) We will follow survival and tumor growth in long term experiments.

The ultimate goal of these studies is to aid in the development of clinical protocols in which ovarian cancer patients will be treated with autologous targeted PBL, monocytes or neutrophils. Toward this end, we are collaborating with Dr. David McKean and coworkers at the Mayo Clinic, who are testing our heteroconjugates against fresh tumor cells from ovarian cancer patients. In addition, we are comparing heteroconjugates with hybrid-hybridoma antibodies, and with hetero-F(ab')<sub>2</sub> antibodies, all obtained from a group in the Netherlands that intends, along with others in Italy and in Switzerland, to begin phase I studies for treating ovarian cancer with targeted T cells. Our studies will impact on the design of their protocols.

## 2. Targeted antigen presentation.

Because a single coat of targeted antigen is efficiently presented to T helper cells, we are hoping that we will be able to follow the course of the antigen within the APC without extraneous noise from irrelevant antigen. In particular, we have established a collaboration with electron microscopists, Drs. Leo Ginsel and Carolina Jost, of the University of Leiden, who have considerable experience following the intracellular trafficking of FcγR. We have several mAb to different sites on HEL, and we are planning to label targeted antigen at various stages of internalization with gold-antibody conjugates, using different anti-HEL antibodies in the hetero and gold conjugates. Preliminary studies have shown that we are able to follow the internalization of antigen, and our current plans are for Dr. Jost to join our laboratory in the middle of next year, and to carry on an extensive electron microscopic study of the internalization and processing of targeted antigen.

We are continuing with our in vivo studies of antigen presentation. Experiments will be done to maximize the enhancement of presentation by heteroconjugates. We are currently initiating studies in which tumor antigens are targeted to APC.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09255-14 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Application of Flow Cytometry to Cell Biology

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

PI: S. O. Sharrow Senior Investigator EIB, NCI

Others: J. P. Henrich Microbiologist EIB, NCI

M. A. Sheard Biologist EIB, NCI

Members of the Experimental Immunology Branch, NCI and

Members of the Immunology Branch, NCI (see text)

COOPERATING UNITS (if any) A. Schultz, CSL, DCRT; L. Barden, CSL, DCRT; R. Tate, CSL, DCRT; J. Bluestone, U. of Chicago; D. DeLuca, Medical Univ. of South Carolina; I. Stroynowski, CALTECH; L Hood, CALTECH; T.A. Waldmann, MB, DCBD, NCI; J.A. Bersofsky, MB, DCBD, NCI; D.H. Sachs, IB, DCBD, NCI.

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

1.0

## OTHER:

2.0

## CHECK APPROPRIATE BOXES)

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

B

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

The Experimental Immunology Branch flow cytometry laboratory currently supports over 80 individual research projects for more than 55 investigators. These investigations involve quantitative single cell analysis of parameters associated with cell types freshly prepared from different species/tissues, as well as spectrum of in vitro cultured cells. Basic research support is provided to members of the EIB and the Immunology Branch, as well as to other members of DCBD. Currently supported projects include a broad range of applications in the areas of cellular immunology, immunochemistry, transplantation biology, clinical immunology, immunogenetics, tumor immunology, and molecular immunology. These include, but are not limited to, the following studies: a) in vivo and in vitro analyses of intra-cellular signalling via T cell surface molecules, b) characterization of T cells from animals with genetic or induced immune dysfunction; c) studies of the pathogenesis of graft-versus-host disease; d) analyses of the correlations between T cell memory and the quantitative expression of cell surface adhesion molecules; e) investigations of T cell ontogeny and differentiation; f) studies of mechanisms of T cell repertoire generation; g) analyses of transplantation antigens and of gene regulation of expression of these antigens; h) investigations involving mechanisms and manipulations of antigen-presentation processes; and i) analyses of the mechanisms involved in marrow graft rejection versus acceptance.

## Project Description

Major Findings:

The EIB flow cytometry laboratory operates and maintains a dual-laser flow cytometer and associated ADP equipment, maintains and provides training for three user-operated single beam flow cytometers, maintains a reagent bank which supplies reagents to members of the EIB and the IB, and provides consultation in flow cytometry techniques, protocol design, reagent selection, and data analysis. This report summarizes findings only in selected project areas which required use of the dual-beam flow cytometer, and emphasizes those aspects most heavily supported by the use of flow cytometry analysis.

Dr. A. Singer and colleagues utilized flow cytometry analysis in a series of investigations involving *in vivo* and *in vitro* analyses of intra-cellular signalling via murine T cell accessory molecules. In one of these studies, anti-CD4 monoclonal antibody was injected *in vivo* to study the effects upon developing T cells of CD4 engagement. It was found that this treatment not only resulted in decreased cell surface expression of CD4, but also affected the expression of cell surface T cell receptor. Further, the cellular response to CD4 engagement, as measured by T cell receptor expression, was found to be dependent upon the maturational stage of the T cells analyzed. Specifically, while CD4 engagement produced decreased receptor expression on mature T cells, immature T cells responded with increased cell surface expression of T cell receptor. These results suggest that CD4 engagement may play an important role in T cell maturation, and have implications for our understanding of the mechanisms by which accessory molecules contribute to T cell maturation and activation. Another series of studies by Dr. A. Singer and colleagues have utilized flow cytometry analysis in investigations of the role of T cell receptor expression in early T cell differentiation and development. In one of these studies, they have analyzed thymocytes from C.B-17/scid mice which are unable to productively rearrange antigen receptor genes and thus generally fail to express T cell receptor. It was found that SCID (severe-combined immunodeficiency disease) thymocytes do not express T cell receptor, CD4 or CD8 and phenotypically resemble a specific normal thymocyte subpopulation which is enriched during embryogenesis, and which is known to contain precursors for mature thymocytes. It was further found that in those occasional thymic which contained small numbers of T cell receptor positive cells, there were also found cells expressing CD4 and/or CD8 accessory molecules. However, T cell receptor and accessory molecules were generally expressed on separate cells within the same thymus. These results suggest that SCID thymocytes, while arrested at an early stage of T cell development, can differentiate further when T cell receptor positive cells are present and that CD4<sup>+</sup>CD8<sup>-</sup>, T cell receptor positive cells, known to occur early in thymus ontogeny, may play an important role in subsequent T cell development.

Dr. Shaw and colleagues have utilized flow cytometry in extending their characterization of cell surface molecules which are differentially regulated on human T cells. Initial investigations had demonstrated that functionally distinct T cell subsets express quantitatively distinct surface levels of several biologically important molecules. Recent studies have focussed on the CD4 positive T cell subset, and involve a systematic analysis of quantitative

surface levels of, and correlations between, more than 200 monoclonal antibodies which react with cell surface products. The results have demonstrated that unexpectedly high numbers of surface antigens are coordinately up-regulated or down-regulated as T cells differentiate between "virgin" (not previously stimulated), and "memory" (previously activated) states of maturation. In addition, the analyses have revealed previously undetected, phenotypically distinct, CD4<sup>+</sup> T cell subsets. These data suggest that phenotypic analysis will allow characterization of additional functionally-distinct CD4 T cell subsets.

Dr. Segal and colleagues have received flow cytometry support in a series of investigations aimed at developing re-targeting methods of enhancing immune responses. In one of these studies, quantitative flow cytometry analysis has been used to investigate the mechanisms by which antigens are bound, internalized, stored, and presented when introduced to antigen-presenting cells with cross-linked bi-functional antibodies. HEL has been introduced into normal murine B cells using anti-HEL antibody chemically-conjugated to antibodies to B cell surface molecules. It has been found that HEL delivered in this manner to surface IgD (using anti-HEL:anti-IgD conjugates) is rapidly and almost completely internalized, followed by storage in low pH, chloroquine sensitive, compartments for up to 20 hours. In contrast, HEL delivered via cell surface MHC molecules (using anti-HEL:anti-Class I or anti-HEL:anti-Class II conjugates) is internalized slowly and does not appear in low pH compartments. However, HEL delivered via MHC molecules is rapidly presented on the surface of B cells in a form which can be recognized by T cells. These results demonstrate that the mechanisms by which B cells process and present antigen are fundamentally different depending upon the route of entry into the cell, and suggest that this process may be manipulated in order to enhance the immune response.

Dr. D. Singer and colleagues have utilized flow cytometry analysis in their investigations of regulation of Class I gene expression. Quantitative flow cytometry has been used to study upregulation of cell surface Class I expression following *in vitro* or *in vivo* treatment with ethanol or with  $\alpha$ , $\beta$ -interferon, and to compare the cell surface expression of endogenous versus transgenic Class I molecules in mice bearing a porcine Class I gene. In one of these studies, cell surface expression of PD1 (transgenic porcine Class I) was compared with that of endogenous murine Class I antigens after *in vivo* treatment with  $\alpha$ , $\beta$ -interferon. It was found that interferon treatment increased cell surface expression of the endogenous classical transplantation antigen, H-2K, but did not affect cell surface expression of Qa-2, a Class I antigen whose expression is limited to hematopoietic cells. Cell surface expression of the transgene product in these interferon treated animals increased in a manner parallel to the endogenous transplantation antigens. These studies demonstrate that Class I gene regulation is precisely controlled in relation to function, even when the gene is expressed in another species.

Dr. Shearer and colleagues utilized flow cytometry analysis in their studies which analyze the pathogenesis of events associated with graft-versus-host (GVH) disease, as well as for investigations of the effects of Cyclosporin A (CSA) on T lymphocyte regulation and development. In one of these studies it was found that when irradiated, syngeneic bone marrow transplanted mice were continuously treated with CSA, the intra-thymic development of CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> (single positive) T cells was completely abrogated. This maturational arrest resulted



in the complete absence of mature T cells in the peripheral lymphoid organs of these mice. In contrast, CSA had no detectable effect upon B cell differentiation and did not affect the intra-thymic development of T cell receptor bearing  $CD4^{-}8^{-}$  (double negative) or  $CD4^{+}8^{+}$  (double positive) T cells. The effects of CSA treatment were also investigated in normal adult animals. It was found that single positive mature T cells ( $CD4^{+}8^{-}$  and  $CD4^{-}8^{+}$ ) disappeared from thymuses of these mice during CSA treatment and reappeared within 2 weeks of termination of CSA treatment. When previously CSA treated animals were irradiated and reconstituted with syngeneic bone marrow, the reconstituted immune system was found to exhibit a striking defect which resulted in the absence of  $CD4^{+}$  T helper function. These functionally deficient mice, however, contained normal numbers of T cell receptor positive,  $CD4^{+}$  T cells. This defect could be reversed by transplantation of a normal (non-CSA treated) thymus, demonstrating that CSA had damaged a component of the thymic micro-environment which is specifically required for development of the functional capacity of  $CD4^{+}$  T cells. These studies not only have important clinical implications for T cell development in transplant patients, but also provide a unique model for investigating mechanisms of T cell development within the thymus.

Dr. Sachs (IB) and colleagues have used flow cytometry analysis to phenotypically characterize and kinetically monitor T cell subset reconstitution in a large series of studies of allogeneic and xenogeneic bone marrow transplantation in irradiated mice and pigs. These investigations employ two general models for bone marrow transplantation; a) lethal irradiation and mixtures of allogeneic (or xenogeneic) marrow containing T cells and syngeneic T-cell-depleted marrow, and b) allogeneic (or xenogeneic) bone marrow only following a preparative regimen employing anti-T cell monoclonal antibody treatment and sublethal irradiation. In one of the murine studies it was found that stable, fully allogeneic, chimerism and specific tolerance, without graft-versus-host disease, could be achieved by pre-treatment of the host animal with a combination of anti- $CD4$  and anti- $CD8$  monoclonal antibodies followed by 300 rads (sub-lethal) whole body irradiation plus 700 rads thymus irradiation. Pre-conditioning host animals with anti-Thy1 monoclonal antibody was not sufficient to produce allogeneic chimerism with this protocol. However, equivalent *in vivo* T cell depletion (phenotypically and functionally) resulted from *in vivo* treatment with either anti-Thy1 antibody or with a combination of anti- $CD4$  and anti- $CD8$  antibodies. It was also found that equivalent results (chimerism and tolerance) were achieved whether or not the fully allogeneic bone marrow was depleted of mature T cells prior to injection. These results demonstrate that the use of *in vivo* monoclonal antibody treatment in transplantation models provides an important tool for investigating mechanisms of alloengraftment, and suggest that it may be possible to develop clinical models for bone marrow transplantation that will achieve alloengraftment in the absence of graft-versus-host disease or radiation-induced toxicity.

Dr. Bluestone (Univ. of Chicago) and colleagues have received flow cytometry support for a variety of investigations involving phenotypic analysis of normal murine T cell subsets and for phenotypic analysis of the effects upon T cells of *in vivo* and *in vitro* treatment with anti- $CD3$  monoclonal antibody. In one of these studies, the cell surface phenotype of peripheral murine  $CD4^{-}, CD8^{-}, TcR\gamma\delta^{+}$  T cells was extensively characterized using three-color fluorescence analysis. It was found that these cells exhibited a phenotype characteristic of normal mature  $TcR\alpha\beta^{+}$  T cells in that they were  $Thy1^{+}, CD3^{+}, CD5^{+}, IL-2R^{-}, HSA^{-}, B220^{-}$ .



Like TcR $\alpha\beta^+$  T cells, a significant percentage (70%) of the TcR $\gamma\delta^+$  T cells expressed the putative memory cell marker Pgp1, suggesting prior responsiveness to antigen exposure *in vivo*. These analyses, combined with functional, biochemical and molecular studies, suggest that CD4 $^-$ , CD8 $^-$ , TcR $\gamma\delta^+$  T cells represent a distinct T cell lineage with *in vivo* function.

S. Sharrow and colleagues have utilized flow cytometry analysis for investigations of Class I antigen expression and epitopes, and for studies of murine T cell differentiation using phenotypic analysis of normal thymocyte subsets and of developing T cells in fetal thymus organ culture. In one of these studies, a new panel of monoclonal antibodies with specificity for the murine Class I-like product Qa-2 was developed. These reagents were used to characterize antigenic determinants on the products of transfected Q genes and to define epitope clusters on the Qa-2 molecule using competitive inhibition analyses. It was found that epitope clusters of the Qa-2 could be divided into three major groups which could be assigned to the 3 protein domains of Class I antigens. It was further found that those anti-Qa-2 reagents with cross-reactivity on other Class I antigens all reacted with an epitope cluster on the first (N) protein domain of Class I, and that previously described anti-Qa-2 specific reagents all reacted with the third (C2) Class I domain. The new panel of reagents also contained antibodies which reacted with an epitope cluster present on the second (C1) Class I domain. In another study, the effects of anti-CD3 treatment upon T cell development in murine fetal thymus organ culture were investigated by phenotypic and biochemical analyses. It was found that anti-CD3 did not abrogate development of CD3 $^+$ , CD4 $^-$ , CD8 $^-$  T cells or of CD4 $^+$ , CD8 $^+$  T cells. In contrast, CD4 $^+$ , CD8 $^-$  T cells could not develop in these anti-CD3 treated cultures. T cells developing in these cultures expressed predominantly  $\gamma\delta$  T cell receptors and were errantly enriched for CD3 $^-$ , CD4 $^-$ , CD8 $^+$  T cells when compared with control cultures. These data suggest that: a) anti-CD3 has different effects upon  $\gamma\delta$  versus  $\alpha\beta$  TcR expressing T cells; b) normal T cell receptor engagement is not required for expression of CD4 and CD8 on double positive T cells. and c) CD3 $^+$ , single positive T cells require appropriate T cell receptor interactions for their development.

S. Sharrow, L. Barden (CSL, DCRT) and colleagues have: a) evaluated and designed methodology and ADP hardware systems for implementing transition to state-of-the-art flow cytometry instrumentation; b) evaluated and designed ADP hardware capabilities for archiving up to 100 gigabytes of list mode flow cytometry data; c) implemented installation in the EIB site of an initial version of the CAP (Cluster Analysis Program) running on the EIB MVAXII; d) established network connections between the development system in CSL, DCRT (Bld. 12A) and the EIB flow cytometry laboratory (Bld. 10) which provides for movement of both software development and data analysis between the two sites; and d) further investigated the application of automated cluster analysis techniques to flow cytometry data. In one of these studies, it has been found that n-plot graphical display, which allows 2-dimensional analysis of n-parameter data, can be successfully used to display raw single cell data. When combined with software gating techniques, this analysis provides information not available using conventional flow cytometry data analysis techniques. This novel approach is being implemented as a feature of the CAP system.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 09256-02 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

In Vitro Study of MHC-Specific T Cells

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

PI: A. Singer Senior Investigator EIB, NCI

Others: P. Stein BTP Fellow EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.15

## PROFESSIONAL:

1.15

## 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 has been aimed at identifying and characterizing the T cell subsets that initiate and effect anti-MHC responses in vitro. We have identified and characterized 3 distinct T helper cell populations that function in vitro to initiate anti-MHC responses, and have studied 3 distinct T killer cell populations that function in vitro as anti-MHC effector cells. We have characterized these cell populations with regard to their specificity, phenotype, activation requirements, and differentiation requirements.

## Project Description

Major Findings:

In contrast to conventional immune responses against foreign protein antigens that involve a single population of Th cells, we have characterized 3 distinct populations of Th cells that function to initiate anti-MHC responses in vitro, based on their recognition of different MHC specificities expressed on the surface of accessory (Acc) cells: a) one Th cell population recognizes class II MHC allodeterminants, b) one Th cell population recognizes class I MHC allodeterminants, and c) one Th cell population recognizes an antigenic complex composed of foreign class I and self-class II MHC determinants. The two class II restricted Th cell populations are phenotypically CD4<sup>+</sup>, whereas the class I restricted Th cell population is phenotypically CD8<sup>+</sup>. One common function performed by these phenotypically distinct anti-MHC Th cell populations was the secretion of IL-2. Furthermore, we found that IL-1 serves as a common co-factor, together with ligand, for triggering both CD4<sup>+</sup> and CD8<sup>+</sup> Th cell populations to secrete IL-2.

To assess the limits of the limited response repertoire expressed by IL-2 secreting CD8<sup>+</sup> Th cells, we examined their ability to respond against a variety of different antigens. We found that CD8<sup>+</sup> Th cells secreted IL-2 in response to MHC class I alloantigens but failed to be activated by self class I presentation of most nominal antigens, such as minor histocompatibility antigens, TNP, etc. However, we found that CD8<sup>+</sup> Th cells could be activated to secrete IL-2 in response to Vaccinia virus, and that such Th cells represented a portion of the normal Th cell pool in virus infected mice. Thus, this is the first demonstration of conventional antigen recognition by CD8<sup>+</sup> Th cells. We interpret these observations as being consistent with the perspective that such cells require a high ligand density for activation, and that these requirements are generally only met by MHC alloantigens and by replicating viral antigens.

To understand the molecular requirements for MHC recognition by class I allospecific T<sub>H</sub> cells, we assessed the role of an exposed portion of the alpha-helical region of a class I MHC molecule in T cell recognition. We found that a peptide identical in sequence to amino acids 163-174 of the Kb molecule blocked T<sub>H</sub> cell recognition of the Kb molecule regardless of the site on the Kb molecule that the T<sub>H</sub> cells were directed against. The blocking of T<sub>H</sub> cell function by peptide Kbl63-174 occurred at the level of the T effector cell, and not at the level of the target cell. Furthermore, we found that peptide Kbl63-174 not only blocked target cell lysis, but also blocked conjugate formation between anti-Kb T<sub>H</sub> cells and Kb-bearing target cells. Taken together, these data indicate that Kbl63-174 blocks T<sub>H</sub> cell engagement of the Kb molecule by binding to the T cells' clonotypic receptor, and consequently, define a docking site on the Kb molecule utilized by most Kb-specific T<sub>H</sub> cells.

Publications:

Mizuochi T, McKean DJ and Singer A. IL-1 as a cofactor for lymphokine-secreting CD8<sup>+</sup> murine T cells. J. Immunol. 1988;141:1571-1575.

Shearer GM, Singer A, Mizuochi T, Buller M, Hugin A and Morse HC. Importance of CD8<sup>+</sup> T helper cell function in AIDS. J. Inf. Dis. 1988;158:893.

Mizuochi T, Hugin AW, Morse DC, Singer A and Buller RML. Role of lymphokine secreting CD8<sup>+</sup> T cells in cytotoxic T lymphocyte responses against vaccinia virus. J. Immunol. 1989;142:270-273.

Munitz TI, Schneck Coligan JE, Maloy WL, Henrich JP, Sharrow SO, Margulies, DH and Singer A. A peptide derived from the alpha-helical region of class I MHC blocks CTL engagement of the class I MHC molecule. In Inglis, J. (Eds.): Cold Spring Harbor Symposia on Quantitative Biology, Volume 54, Immune Recognition. In press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09257-14 E

## PERIOD COVERED

October 1, 1989 to September 30, 1989

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

Mechanisms of Cellular Immune Responses

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

PI:	S. Shaw	Senior Investigator	EIB, NCI
Others:	G. Ginther-Luce	Chemist	EIB, NCI
	Y. Shimizu	Damon Runyer Fellow	EIB, NCI
	K. Horgan	Fogarty Visiting Associate	EIB, NCI
	G. van Seventer	Fogarty Visiting Associate	EIB, NCI
	R. Siraganian	Section Chief	LMI, IRP, NIDR

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

4.0

## 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.)

Analysis of the molecular basis of T cell recognition is currently focused on two fundamental areas: 1) identifying and characterizing the functions of cell surface molecules which facilitate T cell recognition ("accessory molecules"), particularly those which mediate adhesion; and 2) characterization of heterogeneity among subsets of human peripheral blood T cells and determining the functional capacities of the subsets defined.

ICAM-1, which we have previously characterized as a cell surface ligand for LFA-1, has been studied following immunoaffinity purification for its functional role not only in T cell adhesion but also as a costimulus for T cell activation. Its capacity to facilitate CD3-receptor mediated activation, together with other aspects of its regulation and function, suggest that it may be one of the most important "second signals" for T cell activation. Studies of other accessory molecules have identified CD44 as an additional member of the functionally important class of molecules involved both in T cell adhesion and activation. Studies of T cell/ monocyte adhesion are characterizing the molecular basis of this immunologically important cell interaction.

Current studies confirm and extend previous analysis of T cell subsets distinguished by different isoforms of CD45, which we understand to be naive and memory T cells. Methodologic progress allows preparation of very pure subsets whose functional capacities have been studied. Naive cells have markedly reduced proliferative responses to various regimens of receptor-mediated activation; however, their capacity to respond is demonstrated by augmented response in the presence of costimuli such as that provided by IL1/IL6, CD28 or CD44. This suggests important regulatory roles for costimuli in vivo in determining responsiveness of particular subsets. Systematic phenotypic analysis of CD4+ cells with more than 200 monoclonal antibodies reveals unexpected heterogeneity which is being analyzed by multicolor flow cytometry. Such integrated phenotypic and functional analysis is essential to adequately address the functional complexities in T cell responses.



## Project Description

Major Findings:

Although antigen-specific T cell recognition is dependent on the T cell receptor, it is evident that recognition is a complex sequence of interactions involving a number of so-called "accessory molecules". Cell-cell adhesion is an important part of the process, therefore molecules involved in T cell adhesion are being actively investigated. We have previously established (AR09257-13, AR05067-12) that there are two dominant molecular pathways of T cell adhesion which facilitate recognition: T cell CD2 binding to LFA-3 and T cell LFA-1 binding to ICAM-1 and other as yet undefined ligands. Detailed understanding of the role of these receptor/ligand interactions in T cell recognition has been sought using the well-defined model system of T cell interaction with biochemically purified ICAM-1. Emphasis in the last year has been on testing the hypothesis that ICAM-1 functions not only as a ligand for LFA-1-dependent adhesion but also in providing a costimulus which facilitates T cell activation. Our results demonstrate that T cell binding to ICAM-1 is associated with a dramatic enhancement of receptor-mediated T cell activation. The exact details of the enhancement depend on the model system used. T cell responses to triggering anti-T cell receptor (CD3) monoclonal antibody (mAb) immobilized on plastic is strongly augmented when the ICAM-1 is co-immobilized on the plastic; this augmentation is in addition to affects mediated by IL1/IL6 and IL2. T cell responsiveness to activating pairs of CD2 mAb, another kind of receptor-mediated activation, is also augmented by ICAM-1, but only in the presence of IL1/IL6. This data indicate that the ICAM-1 molecule facilitates T cell activation, apparently by providing a co-stimulus which augments signal generation by the T cell receptor. Together with other data regarding: 1) the singular effectiveness of activated monocytes in T cell stimulation, 2) the upregulation of ICAM-1 with monocyte activation, and 3) the role of monocyte ICAM-1 in T cell stimulation, these data strongly suggest that ICAM-1 provides a physiologically important "2nd signal" for T cell activation.

Given the importance of T cell interactions with monocytes, detailed analysis of T cell binding to monocytes has been undertaken with more than 200 mAb from the 4 International Workshop on Leukocyte Differentiation Antigens. Analysis of the results demonstrates clear classification of mAb of the CD11a/CD18 family which are involved in mediating this adhesion. Further analysis is in progress of other molecules potentially involved in this adhesion.

Seeking to identify additional accessory molecules by screening for monoclonal antibodies (mAb) which inhibit T cell adhesion to erythrocytes, we have identified a functionally unique CD44-specific mAb, NIH44-1, which partially inhibits T cell rosetting by binding to CD44 on the erythrocyte. NIH44-1, which immunoprecipitates a protein of 85-110 kD with broad tissue distribution, was determined to be specific for CD44 based on comparison of its tissue distribution with multiple CD44-specific reference mAbs and sequential immunoprecipitation with such mAbs. Anticipating a role for many adhesion

molecules in signal transduction, we studied the effect of CD44 mAb on T cell activation and observed that CD44 mAb dramatically augments T cell proliferation induced by CD3- and CD2-receptor-mediated activation. The augmentation of the response to immobilized CD3 mAb by exhaustively monocyte-depleted T cells indicates that augmentation can be mediated by binding to the T cell. Thus, our studies demonstrate specific new roles for CD44 in T cell adhesion and activation. Furthermore, we suggest that: 1) CD44 has a role in adhesion of cells of multiple lineages; and 2) CD44 may participate in adhesion not (only) by functioning as an adhesion receptor but rather by serving as an anchorage site for other adhesion molecules.

Our previous studies have demonstrated that a group of functionally important cell surface molecules are differentially expressed on subsets of human peripheral T cell subsets which we interpret to be naive and memory cells. (AR09257-13). Given the potential importance of this observation, the studies have been extended in two directions: further functional characterization of those subsets, and exploration for other molecules differentially regulated on peripheral blood CD4<sup>+</sup> cells. CD4<sup>+</sup> naive (CD45RO<sup>-</sup>) and memory (CD45RA<sup>+</sup>) cells were rigorously purified by immunomagnetic negative selection and studied for differences in CD3 receptor-mediated activation. Naive cells proliferate more poorly than memory cells to anti-CD3 mAb in the presence of monocytes; this defect is seen irrespective of the anti-CD3 mAb and donor studied, and contrasts with comparable PHA-responsiveness of both subsets. Poorer naive cell responses are also observed when the anti-CD3 mAb is immobilized on plastic in the absence of monocytes; this suggests an intrinsic hyporesponsiveness of naive cells, independent of their potentially weaker interaction with monocytes. Naive cells also proliferated less well than memory cells to Staphylococcus Enterotoxins A and B over a wide dose range in the presence of monocytes; such "superantigens" are thought to mimic antigen-specific stimulation but to have the experimental advantage of stimulating a higher frequency of T cells (due to exclusive TCR V $\beta$  interaction). The hyporesponsive of naive cells could be overcome by costimuli including CD28 mAb, CD44 mAb or a combination of IL1 and IL6. The pattern of augmentation was dependent on the particular triggering regimen. Thus, although CD4<sup>+</sup> naive cells are less responsive than memory cells to activation signals via the CD3 receptor complex, strong responses can be elicited from naive cells when appropriate costimuli are supplied. We interpret this to indicate that naive cells are capable of antigen response, and such responses will be regulated by the available costimuli in vivo.

Our expectation is that molecules which are differentially regulated on T cell subsets are likely to be critical to their differentiated function. This general tenet has been reinforced by our previous results with naive and memory cells. Therefore, we have extended analysis by systematic studies of binding of more than 200 mAbs to CD4<sup>+</sup> peripheral T cell using multicolor flow cytometry. As a result of our methodologic improvements and systematic analysis, much more complexity is observed than has previously been appreciated. Further analysis is in progress in order to correlate markers with each other; this will allow us to discern how many distinct subsets are present and which markers are coordinately regulated on those subsets. Not only will this analysis provide a starting point for studies of the molecules differentially regulated, and for further functional analysis of heterogeneity of T cell function, but also it will provide an important ensemble of markers with which to study clinical diseases.

Publications:

Shaw S, Luce GG, Springer TA, Plunkett ML, Quinones R, Gress RE, Sanders ME. Human cytotoxic T-cells adhere to potential targets by two antigen-independent pathways: CD2 binding to LFA-3 or LFA-1 binding to an undefined ligand. In: McMichael AJ, Beverley PC, Cobbold S, et al, eds. Leucocyte type III. White cell differentiation antigens. New York: Oxford University Press, 1987;137-139.

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Selvaraj P, Plunkett ML, Dustin M, Sanders ME, Shaw S, Springer TA. The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. Nature 1987; 326:400-403.

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Makgoba MW, Sanders ME, Luce GE, Dustin ML, Mannoni P, Clark EA, Springer TA, Shaw S. A cluster of antibodies (RR1/1, LB-2, and 84H10) which inhibit LFA-1-dependent lymphoid and myeloid cell adhesion bind intracellular adhesion molecule-1 (ICAM-1). In: Dupont B, ed. Immunobiology of HLA. Vol #2 Immunogenetics and histocompatibility. New York: Springer-Verlag, 1989;577-580.

Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S. Coordinate enhanced expression of three adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (4B4, UCHL1, and Pgp-1) defines a human T cell subset containing memory cells and characterized by enhanced gamma interferon production. In: Dupont B, ed. Immunobiology of HLA. Vol #2 Immunogenetics and histocompatibility. New York: Springer-Verlag, 1989;560-562.

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Hall RP, Sanders ME, Duquesnoy RJ, Katz SI, Shaw S. Alterations in HLA-DP and -DQ antigen frequency in patients with dermatitis herpetiformis. *J Invest Dermatol* 1989; in press

Biddison WE, Shaw S. CD4 expression and function in HLA class II-specific T cells. *Immunol Rev* 1989; in press.

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Horgan KJ, Shaw S. Immuno-magnetic negative selection of lymphocyte subsets. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds. *Current Protocols in Immunology*. : 1989; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09258-11 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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 Senior Investigator EIB, NCI

Others: M. Vacchio Bio. Lab. Tech EIB, NCI

J. Berzofsky Senior Investigator MB, NCI

## COOPERATING UNITS (if any)

Alison Finnegan, Rush-Presbyterian Saint Lukes, Chicago, IL

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.1

## OTHER:

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.)

In order to analyze the genetic regulation of T cell responses to NASE, a series of cloned lines were generated in BALB/c (H-2<sup>d</sup>) as well as (H-2<sup>b</sup> x H-2<sup>a</sup>)F<sub>1</sub> T cells. Individual clones were restricted to recognizing NASE in the context of either A<sub>α</sub>A<sub>β</sub> or E<sub>α</sub>E<sub>β</sub> 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<sup>b</sup>A<sup>b</sup><sub>β</sub> restricted clones were selectively responsive to peptide 91-110; E<sup>k</sup>E<sup>k</sup><sub>β</sub> restricted clones were responsive to peptide 81-100.

The nature of the immunogenic peptides which are processed and presented to T cells was further evaluated using a panel of variant NASE peptides. It was observed that negative (inhibitory) interactions appear to occur between amino acids in peptides which interfere with T cell responses. Single amino acid changes, by eliminating such apparent negative interactions, result in the restoration of T cell stimulatory ability in these peptides.

## Project Description

Major Findings:

Monoclonal T<sub>H</sub> cell populations specific for NASE have been generated in BALB/c (H-2<sup>d</sup>) and (B10xB10.A)F<sub>1</sub> (H-2<sup>b</sup>xH-2<sup>a</sup>) 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<sub>1</sub> clones respond to native NASE in association with either A<sup>b</sup><sub>α</sub>A<sup>b</sup><sub>β</sub> or E<sup>k</sup><sub>α</sub>E<sup>k</sup><sub>β</sub>. 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<sup>b</sup><sub>α</sub>A<sup>b</sup><sub>β</sub> restricted clones were highly responsive to peptide 91-110; and not to other synthetic NASE peptide. In contrast, E<sup>k</sup><sub>α</sub>E<sup>k</sup><sub>β</sub> restricted clones were consistently responsive to peptide 81-100 and not to 91-110; certain of these E<sup>k</sup><sub>α</sub>E<sup>k</sup><sub>β</sub> restricted T cells expressed a cross reactivity with peptide 51-70. A BALB/c clone was responsive only to peptide 61-80. No other NASE peptides have revealed antigenic activity to date. Collectively, these findings demonstrate that NASE specific T cells are responsive to discrete peptides. Moreover, these findings suggest that in T cell recognition of a complex and highly foreign protein antigen, a limited number of peptide epitopes is preferentially recognized by T cells in association with a given Ia molecule.

More detailed analysis of T cell fine specificity in response to NASE peptides was carried out using variants in which a single amino acid was altered from its native or wild type form. This analysis revealed that clones with specificity for the same native peptide and self MHC determinant may nonetheless vary significantly in their fine specificity as assessed by responses to variant peptides. It was also observed that certain E<sup>k</sup><sub>α</sub>E<sup>k</sup><sub>β</sub> restricted clones responded to peptide 91-100 but not to 91-105. On the basis of this finding, it was suggested that interactions between amino acids in the 101-105 region with amino acids in the stimulatory 91-100 region were responsible for impaired ability to stimulate T cells. Consistent with this hypothesis, it was found that single amino acid changes including those in the 91-100 as well as those in the 101-105 region were capable of restoring stimulatory ability. Such changes appear to have their effect by eliminating inhibitory interactions among amino acid residues in the 91-105 peptide.

Proposed Course of Project:

Cloned T cell populations and synthetic peptides will be employed to further analyze the mechanism for antigen fine specificity and MHC restriction. Variant peptides will be analyzed to more definitively identify antigenic sites on peptides antigens.

The influence of non-MHC genes on anti-NASE responses will be studied. As noted in project Z01 CB 05106-07, expression of non-MHC Mls determinants can result in deletion of expression of certain Vβ genes. The influence of non-MHC genes, including Mls<sup>a</sup> and Mls<sup>c</sup>, will therefore be studied in T cell responses to NASE. Both the peptide fine specificity of these responses and the patterns of T cell receptor Vα and Vβ expression will be used to monitor the expressed repertoire by both functional and structural criteria.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09259-11 E

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Effects of Graft-versus-Host Reactions on Cell-Mediated Immunity

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

PI: G. M. Shearer Senior Investigator EIB, NCI

Others: F. Hakim Senior Staff Fellow EIB, NCI  
 S. Sharrow Senior Investigator EIB, NCI

COOPERATING UNITS (if any)

A. Rosenberg, Dept. of Biologic Testing, FDA, Bethesda, MD  
 C. S. Via, Dept. of Medicine, University of Maryland, School of Medicine,  
 Baltimore, MD

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

2.5

OTHER:

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.)

Inoculation of parental T cells into F<sub>1</sub> mice, which induces a graft-versus-host (GVH) reaction, can result in different immune abnormalities, depending on the donor and host strains used. Injection of C57BL/6 cells into B6D2F<sub>1</sub> mice resulted in extensive immune suppression, hypogammaglobulinemia, and susceptibility to infection. Injection of DBA/2 cells into B6D2F<sub>1</sub> mice resulted in selective suppression of L3T4<sup>+</sup> T helper cell function, hypergammaglobulinemia, and autoantibody production with SLE-like symptoms. The differences in these two forms of SLE were attributed to a defect in DBA/2 anti-F<sub>1</sub> CTL precursor frequency. Female mice undergoing GVH induced SLE appear to have more severe disease than male mice.

The parent-into-F<sub>1</sub> GVH reaction also results in severe defects in bone marrow stem cell function. Thus, both the levels of IL-3 and CSF were reduced in GVH mice.

Furthermore, the parent-into-F<sub>1</sub> GVH can result in a defect in self-restricted, CD4-mediated T helper cell function, but not in CD8-mediated T helper effector function. This defect is the result of a GVH-induced defect in the thymus.

## Project Description

Major Findings:

F<sub>1</sub> 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<sub>1</sub>. GVH induced by parent anti-host class II only results in loss of self + X responses, but not in loss of allogeneic responses, and is associated with a selective loss of CD4<sup>+</sup> T helper cell function and of CD4<sup>+</sup> T cells. In contrast, class I + II-induced GVH resulted in loss of self + X and allogeneic responses, and was associated with a loss of CD4<sup>+</sup> and Lyt2<sup>+</sup> T helper cell function. The induction of class II only GVH required activation of only CD4<sup>+</sup> parental cells, whereas induction of class I + II GVH required activation of both CD4<sup>+</sup> and CD8<sup>+</sup> cells.

Several parallels have been established between AIDS 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 GVH and in the early stages of AIDS, self + X responses are lost whereas MHC allogeneic responses remain intact. Furthermore, the developmental stages of AIDS and class II GVH result in a loss of CD4<sup>+</sup> T helper cells. It is also noteworthy that susceptibility to certain pathogens in both AIDS and in class II GVH requires not only the loss of the "T4+" helper cell population, but also "T4-" T helper cells. This implies that the "T4-" helpers are relevant for immune responses to antigens other than MHC alloantigens -- and to pathogenic agents in particular. Another parallel between AIDS development and class II GVH is the polyclonal activation of B cells and autoantibody production. Thus, it may be that the very early immune signals that occur after HIV infection are similar to the immunostimulatory model of GVH, as well as those observed in autoimmune mouse strains.

We have also observed that certain class I + II GVH situations result in functional defects that resemble class II GVH only, i.e., in a selective loss of self + X, but retention of all reactivity. These examples of GVH appear to be auto-immune-like, and result in the production of anti-DNA antibodies and a lupus-like syndrome. The example that we have studied is DBA/2 into B6D2F<sub>1</sub> (compared with C57BL/6 into B6D2F<sub>1</sub>). The B6 GVH model results in hypogammaglobulinemia, profound immune deficiency that is not circumvented by IL 2 in vitro, suppressor cell activity that affects both CD4<sup>+</sup> and CD8<sup>+</sup> cell function, and susceptibility to immune cytomegalovirus infection. In contrast, the DBA/2 GVH model results in hypergammaglobulinemia, anti-DNA antibodies, a deficiency and suppressor cell activity that is limited to CD4<sup>+</sup> T helper cell function and that is circumvented in vitro by IL 2, and resistance to cytomegalovirus infection. B10.D2 spleen cells into B6D2F<sub>1</sub> resembles the B6 GVH model indicating the different forms of GVHID and not H-2 linked. Studies with (BxD) R. I. lines indicate that the difference is due to more than one non H-2 linked gene. The functional difference between DBA and B6 that we consider to be responsible for the two different forms of GVHID is that DBA/2 spleens contain 9-fold fewer anti-F<sub>1</sub> CTL precursors than to B6 spleens. Furthermore, the DBA GVH could be converted to the B6 GVH by increasing the inoculum size, and the B6 GVH could



be converted to the DBA GVH by depletion of B6 CD8<sup>+</sup> cells. These findings suggest that this model of lupus is due to a deficiency in CD8<sup>+</sup> effector cells that would normally remove the source of antigenic stimulation and IL 2 production. Thus, a fundamental defect in SLE patients could be deficient effector function for the relevant antigen which leads to continuous and unopposed antigenic stimulation. In this autoimmune model of GVH, female mice appear to get more severe disease than male mice, which is consistent with the observation that SLE is 9-times more prevalent in women than in men.

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. Furthermore, GVH can result in depressed production of IL 3 and GM-CSF, which are important cytokines for hematopoiesis. The ability of the thymus of GVH mice to induce stem cells to mature into CD4<sup>+</sup> T helper cells is abrogated, but the ability of the thymus of the same GVH mice to induce differentiation of allospecific T helper cells remains intact. This defect appears to involve that component of the thymus that is responsible for MHC self-restriction and the discrimination between self and non-self.

#### Publications:

Fukuzawa M, Shearer GM. Effect of graft-versus-host reaction on thymic function. *Immunol. Res.* 1988;7:239-246.

Via CS, Shearer GM. Murine graft-versus-host disease as a model for the development of autoimmunity: relevance of cytotoxic T lymphocytes. *N.Y. Acad. Sci.* 1988;532:44-50.

Fukuzawa M, Shearer GM. Selective L3T4<sup>+</sup> T helper defect for the generation of cytotoxic effector cells in graft-versus-host, bone marrow reconstituted mice. *N.Y. Acad. Sci.*

Hakim FT, Shearer GM. Immunological and hematopoietic deficiencies of graft versus host disease. In J.L.M. Ferrara, and S.J. Burakoff (Eds.): *Graft Versus Host Disease 1989*, Marcel-Dekker:New York, in press.

Fukuzawa, M, Shearer GM. Defective thymic education of L3T4<sup>+</sup> T helper cell function in graft-versus-host mice. *J. Immunol.* 1988;141:430-439.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09260-09 E

## PERIOD COVERED

October 1, 1989 to September 30, 1989

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

Synergistic Effects of Murine Cytomegalovirus and Graft-versus-host Reaction

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

PI: G. M. Shearer Senior Investigator EIB, NCI

Others: S. Sharrow Senior Investigator EIB, NCI

## COOPERATING UNITS (if any)

J. D. Shanley, V.A. Hospital, Newington, CT  
 C. S. Via, Dept. of Medicine, University of Maryland, School of Medicine,  
 Baltimore, MD

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.1

## OTHER:

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.)

Injection of F<sub>1</sub> hybrid mice with either murine cytomegalovirus (MCMV) or parental spleen cells (graft-versus-host reaction - GVHR) results in rapid and severe immunosuppression. Inoculation of either the virus or parental cells were selected so that they would be below the threshold for severe immunosuppression. However, when these two inocula were combined, severe immunosuppression was observed. Furthermore, injection of F<sub>1</sub> mice with parental lymphocytes that recognize only class I MHC determinants does not result in immune suppression. However, a combination of class I recognition and MCMV infection results in profound immune suppression. In contrast, there was no detectable synergy between class II GVH and MCMV. Infection of host mice with MCMV prior to induction of GVH resulted in augmented immune suppression, whereas infection of donor mice with MCMV before induction of GVH resulted in reduced immune suppression. The combination of MCMV and GVHR also resulted in interstitial pneumonitis, whereas either insult alone had no detectable pathogenic effect on the lungs. These studies permit the investigation of the immunosuppression of MCMV infection and the possibility consequences of CMV infection coupled with a GVHR. The anti-viral drug, DHPG, inhibited viral replication but did not prevent MCMV from synergizing with GVH.

Lethally irradiated mice grafted with syngeneic bone marrow develop sarcomas between 18 and 24 months after irradiation. Mice infected with MCMV two weeks after irradiation did not develop tumors, which may suggest that immunity to MCMV may protect mice against developing irradiation-induced sarcomas.

## Project Description

Major Findings:

To determine whether the MCMV-GVH synergy was more pronounced when MCMV was induced by donor leukocytes or was resident in the host at the time of GVH induction, prospective donors of parental spleen cells were infected with MCMV at various times prior to injection into F<sub>1</sub> hosts of GVHR were infected with MCMV at different times prior to injection of parental cells. The results indicate that MCMV infection not only reduces the number of parental spleen cells required to induce graft-versus-host immune deficiency (GVHID) but accelerates the onset of GVHID, which occurs as early as 3 days after cell and virus challenge. To determine whether MCMV infection exerts this synergistic effect of primarily through the donor or the host component, we examined the effect of MCMV infection of either donor mice or recipient mice at 3, 10 and 17 days prior to spleen cell transfer. When donor mice were infected with MCMV three days prior to cell transfer, the ability of donor cells to induce GVHID was reduced. In contrast, MCMV infection of the recipients three days prior to cell transfer increased their susceptibility to GVHID induction. Infection of either donor or host mice 10 days or 17 days prior to parental spleen cell transfer has little effect on the ability to either induce or resist GVHID when compared to sham infected mice. Thus, acute MCMV infection can modulate the severity of GVHID, depending on whether it is the donor or the host that is infected. The ability of acute MCMV to alter the course and severity of GVHID may be relevant for human bone marrow transplants in which preceding CMV infection has been associated with chronic GVH. In this setting, CMV may lower the threshold necessary to induce a GVH reaction.

Our observations that a) MCMV infection is often associated with enhanced T effector cell immunity to non-CMV antigens; and b) MCMV can synergize with low doses of donor leukocytes to induce GVH disease raises the possibility that MCMV infection can initiate a T helper signal with the production of lymphokines. Such a helper signal must be generated by MCMV-specific, MHC self-restricted helpers with the production of IL-2 (and other T helper factors), which could enhance the CD8 component of T cell immunity. A class I + II GVHR results in the loss of both CD4 and CD8 T function, whereas a class II GVH results in a selective loss of CD4 T helper function. A class I GVH that is limited to recognition of H-2D<sup>b</sup> or H-2D<sup>d</sup> results in no detectable GVH, as determined by host T cell function. We have used the differential effects of these three types of GVH on T cell immune function to determine whether CMV infection is providing a "class I" or "class II" type of signal in its synergistic action with GVH, i.e., with or without CMV the class II GVH abrogated only CD4 function. In contrast, when MCMV was given in conjunction with the H-2D GVH (which had no detectable effect alone), the combination of MCMV and H-2D class I recognition abrogated both CD4 and CD8 T cell function - characteristic of a class I + II GVH effects of MCMV recognition by T cells in vitro may elucidate the immune mechanisms responsible for the augmenting effects of MCMV in synergy with GVH, and in other models of immune enhancement.

In some experiments we have attempted to design protocols in which lethally irradiated mice were transplanted with allogeneic bone marrow with or without T cells and with or without CMV infection. Such experimental designs would more closely approximate the situation encountered in human marrow transplantation. Due to the high incidence of mortality in CMV-infected, GVH, irradiated mice, these complex experiments have not been successful. However, the mice that survived long-term were those that were irradiated and grafted with syngeneic bone marrow, with or without CMV infection. Between 12 and 18 months after irradiation and syngeneic marrow grafting, these mice began to develop sarcomas, probably as a result of exposure to 900R of whole-body irradiation. However, the development of the sarcomas (5/11) appeared to be limited to those mice that received 900R, and syngeneic marrow, but not MCMV. Mice that were exposed to MCMV, in addition to receiving irradiation and syngeneic marrow did not develop detectable tumors (0/17). Although these results (compilation of two experiments) are incomplete and need to be redesigned and repeated, the preliminary observations raise the possibility that exposure to MCMV has provided some element of immune protection against the development of the sarcomas. One possibility is that the mechanism responsible for enhanced, immune reactivity of MCMV-infected mice noted above could contribute to the prevention or delayed development of these radiation-induced tumors.

#### Publications:

Via CS, Shanley JD, Weatherly BR, Lang P, Shearer GM. Altered threshold for the induction of graft-versus-host immunodeficiency following murine cytomegalovirus infection: Host and donor contributions. Transplantation 1988; 46:298-302.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09263-08 E

## PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structure and Function of Cytotoxic and Helper T lymphocyte Granules

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

PI: P. A. Henkart Senior Investigator EIB, NCI

Others: K. Andrews Microbiologist EIB, NCI  
M. Hayes IRTA Fellow EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.5

## OTHER:

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.)

Purified CTL and NK cytoplasmic granules contain a "nuclear DNA releasing activity" assayed by their ability to cause release of labelled DNA from detergent solubilized nuclei. The activity was inactivated by the protease inhibitors PMSF and aprotinin. This activity was purified from the extracts of purified CTL granules by gel filtration and affinity chromatography on a aprotinin. The purified preparation had a single protein band with a reduced MW of 30kd, and non-reduced MW of 60kd, consistent with the known behavior of granzyme A. The activity was bound by antibodies against granzyme A, and behaved as a monomer on gel filtration after reduction and alkylation along with granzyme A. These results show that granzyme A can trigger a DNA breakdown process in detergent permeabilized cells. Purified cytolysin was found to permeabilize cells to allow granzyme A mediated DNA breakdown, as would be required according to the granule exocytosis model. The mechanism of this granzyme A induced nuclear breakdown was probed in studies showing that washed detergent extracted nuclei (plus cytoskeleton) did not release DNA in the presence of purified granzyme A, but did so if soluble detergent cell extract was added back. Pre-incubation of the nuclei with granzyme A followed by addition of the soluble extract resulted in DNA release; these experiments show that granzyme A digests a nuclear component, allowing the cytoplasmic fraction to cleave DNA.

## Project Description

Major Findings:

In attempting to reconcile the granule exocytosis model for lymphocyte mediated cytotoxicity with the phenomenon of target cell DNA breakdown, we have tested the ability of components of granules from cytotoxic lymphocytes to breakdown nuclear DNA. Purified CTL and NK cytoplasmic granules contain a "nuclear DNA releasing activity" assayed by their ability to cause release of labelled DNA from detergent solubilized nuclei. The activity was inactivated by the protease inhibitors PMSF and aprotinin. This activity was purified from the extracts of purified CTL granules by gel filtration and affinity chromatography on aprotinin, while also monitoring for the BLT-esterase activity of granzyme A. These two activities co-purified at each step. The final preparation had a single protein band with a reduced MW of 30kd, and non-reduced MW of 60kd, consistent with the known behavior of granzyme A. The activity was bound by antibodies against granzyme A, and behaved as a monomer on gel filtration after reduction and alkylation along with granzyme A. These results show that granzyme A can trigger a DNA breakdown process in detergent permeabilized cells. Purified cytolysin was found to permeabilize cells to allow granzyme A mediated DNA breakdown, as would be required according to the granule exocytosis model. The mechanism of this granzyme A induced nuclear breakdown was probed in studies showing that washed detergent extracted nuclei (plus cytoskeleton) did not release DNA in the presence of purified granzyme A, but did so if soluble detergent cell extract was added back. Pre-incubation of the nuclei with granzyme A followed by addition of the soluble extract resulted in DNA release; these experiments show that granzyme A digests a nuclear component, allowing the cytoplasmic fraction to cleave DNA.

Proposed course:

The mechanism of this phenomenon is still unclear. Specifically, we would like to ascertain the physiological substrate of granzyme A which is responsible for its effect in allowing nuclear DNA release. In a preliminary experiment we have labelled target cell proteins with <sup>35</sup>S-methionine and incubated the detergent insoluble fraction of these cells with granzyme A. Several proteins were found to be degraded by the enzyme. We would like to identify these proteins, as good protein substrates for granzyme A have not been previously described. Similarly, the active component of the soluble cell extract which mediates DNA breakdown is unknown. The simplest explanation consistent with the above findings is that there is a soluble nuclease which acts on DNA after granzyme A removes a protein bound to the DNA. This hypothesis will be tested.

Publications:

Munger WE, Berrebi GA and Henkart PA. Possible involvement of CTL granule proteases in target cell DNA breakdown. *Immunol. Rev.* 1988;103:99-109.

Henkart PA. Lymphocyte Cytotoxic Mechanisms. In: *Immunophysiology: Role of Cells and Cytokines in Immunity and Inflammation*, edited by Oppenheim JJ and Schevach, EM: Oxford University Press. New York, 1988;p 1-33.

Taplits MS, Henkart PA and Hodes RJ. T helper cell cytoplasmic granules: Exocytosis in response to activation via the T cell receptor. *J. Immunol.* 1988;141:1-9.

Quinones RR, Segal DM, Henkart P, Perez P, Gress RE. Inhibition of cytotoxic T cell lysis by anti-CD8 monoclonal antibodies: Studies with CD3-targeted cytolysis of nominal antigen-negative targets. *J. Immunol.* 1989;142:2200-2206.

Hayes MP, Berrebi GA, Henkart PA. Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. *J. Exp. Med.* 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09264-02 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Effects of Cyclosporin A on T Lymphocyte Regulation and Development

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

PI: G. M. Shearer Senior Investigator EIB, NCI

Others: S. Sharrow Senior Investigator EIB, NCI

A. Kosugi Visiting Fellow EIB, NCI

M. Clerici Visiting Fellow EIB, NCI

A. Palestine Senior Investigator CB, NCI

## COOPERATING UNITS (if any)

P. Kimmel, Renal Unit, George Washington University, School of Medicine

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Our laboratory has investigated the effects of CSA on peripheral T cell immune function, as well as on the ability of CSA-treated mice to develop functional T cells from bone marrow stem cells. Multiple injections of different doses of CSA had different effects on peripheral T cell function. The lower dose of CSA selectively abrogated CD4 T helper cell function, but not CD8 T helper or effector function. Suppressor cells were detected that suppressed the CD4 but not CD8 T cell function of normal mice. We have recently obtained similar results by either exposing lymphocyte from healthy humans to different doses of CSA, or by testing the lymphocyte of kidney allografted patients on CSA therapy. Mice treated with these doses of CSA were unable to develop CD4 T helper cell function from normal stem cells, and this defect was due to CSA-induced thymic damage. These results indicate that abrogation of T cell function by CSA can be selective, depending on the dose of CSA administered. Administration of CSA to mice also prevented the development of mature T cells in the thymus and peripheral immune tissues.



## Project Description

Major Findings:

Mice exposed to different daily doses of CSA of 30 or 75 mg/kg/day were tested for in vitro generated splenic T cell responses to antigens that require CD4 T helpers (TNP-self and CD4-mediated ALLO) and to those that can use CD8<sup>+</sup> T helpers (ALLO). Functional tests performed one day after termination of CSA indicated that 30 mg/kg CSA selectively abolished CD4 T helper function whereas 75 mg/kg CSA abrogated all T helper and effector function. Immune function returned within 2 weeks of termination of CSA. At the 30 mg/kg dose, radio-sensitive suppressor T cells of the Lyt2 phenotype were detected which selectively suppressed CD4<sup>+</sup> T helper function of normal syngeneic spleen cells. At 75 mg/kg CSA, radioresistant, non-T suppressor cell activity was detected that suppressed both CD4 and CD8 function of normal spleen cells. That this "suppressor phenomenon" induced by the 30 mg/kg dose was due to cells with suppressor activity and was not due to the trivial explanation of "CSA carryover" was demonstrated by the fact that suppressor activity was radiosensitive, and abrogated by anti-Thy 1.2 or anti-Lyt2 antibodies and complement. These findings indicate that different subsets of T cells can be differentially affected by CSA, depending on the dose used which could account for the failure of some studies to detect in vitro functional effects of CSA. Knowledge of our findings could be important in designing protocols for allograft patients. Our observations that the immune deficiency induced by CSA is associated with suppressor cell activation should be important in elucidating the mechanism(s) responsible for the CSA effect on preventing graft rejection.

In vitro studies in which different doses of CSA were added to primary sensitization cultures of murine spleen cells and human PBL indicate a similar pattern. Thus, lower doses of CSA selectively abrogated CD4-mediated helper function, whereas higher doses of CSA abolished all helper function. These results demonstrate that a similar CSA dose-dependent selective effect on CD4<sup>+</sup> T helper function exists for human T cells.

The GVH effects that we observed on thymic potential to develop CD4<sup>+</sup> T helper cell function, and the effects of CSA reported on T cell development in irradiated marrow-reconstituted animals suggested that CSA treated mice that are subsequently irradiated and marrow grafted (CSA-BMT) would fail to develop CD4<sup>+</sup> T helper cell function. This prediction was confirmed, as CSA-BMT mice exhibited normal CD8<sup>+</sup> T helper and effector function, but failed to show CD4 T helper function. Similar to the GVH-BMT mice described above, the phenomenon: a) was not associated with suppressor cells; b) was not due to a defect in APC function; c) was not associated with a reduction in L3T4<sup>+</sup> or Lyt2<sup>+</sup> cells nor T3 expression; d) was circumvented in vitro by T cell helper factors or L3T4<sup>+</sup> cells from untreated mice; and e) was corrected in vivo by a neonatal thymus graft. These findings of the effects of CSA on thymic function complement studies of others, indicate that CSA can have a profound effect on the thymus, and provide another model for investigating the role of the thymus on T cell functional development.

CSA also prevented the development of mature  $L3T4^+ Lyt2^+$  and  $L3T4^-Lyt2^+$  T cells in the thymus and spleens of mice. Our results indicate that CSA blocks T cell maturation steps in the development of double positive thymocytes from double negative precursors, as well as interfering with the maturation of single positive T cells from double positive precursors.

Publications:

Fukuzawa M, Shearer GM. Effect of cyclosporin A on T cell immunity. I. Dose-dependent suppression of different murine T helper cell pathways. Eur. J. Immunol. 1989;19:49-56.

Kosugi A, Sharrow SO, Shearer GM. Effect of cyclosporin A on lymphopoiesis. I. Absence of mature T cells in thymus and periphery of bone marrow transplanted mice treated with cyclosporin A. J. Immunol. 1989;142:3026-3032.

Fukuzawa M, Sharrow SO, Shearer GM. Effect of cyclosporin A on T cell immunity. II. Defective thymic education of CD4 T helper cell function in cyclosporin A-treated mice. Eur. J. Immunol. 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09265-08 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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 Senior Investigator EIB, NCI

Others: R. Abe Investigator EIB, NCI

M. Vacchio Bio. Lab. Tech. EIB, NCI

## COOPERATING UNITS (if any)

Food and Drug Administration

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.0

## OTHER:

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 nature of polymorphism and allelism were re-evaluated in the minor lymphocyte stimulating (Mls) system. Mls determinants are defined by the ability to stimulate primary proliferative T cell responses between major histocompatibility complex (MHC) identical cells. Originally, Mls was described as a single locus system involving at least four polymorphic alleles. Proliferating T cell clones were generated which were specific for Mls<sup>a</sup>, Mls<sup>c</sup>, or Mls<sup>d</sup>. These, in combination with primary proliferative T cell responses, were then employed to analyze the relationship between Mls<sup>a</sup>, Mls<sup>c</sup>, and Mls<sup>d</sup> determinants. Formal genetic analyses revealed that Mls<sup>a</sup> and Mls<sup>c</sup> are encoded by non-allelic and in fact unlinked genes. Moreover, an Mls<sup>d</sup> strain expresses independently the products of unlinked Mls<sup>a</sup>-like and Mls<sup>c</sup>-like genes. Thus, in contrast to previous understanding, the Mls system is composed of the products of at least two unlinked loci, with no evidence for structural polymorphism at either locus at the present time.

An analysis of T cell receptor (TCR) expression in Mls<sup>c</sup> reactive T cells demonstrated a striking association of this reactivity with expression of the V $\beta$ 3 gene product. In addition, it was demonstrated that mouse strains which express Mls<sup>c</sup> delete expression of V $\beta$ 3 in their mature peripheral T cell populations. This failure to express V $\beta$ 3 presumably reflects a consequence of the maintenance of tolerance to self Mls<sup>c</sup> products. These findings provide a basis for the understanding of high T cell precursor frequency for Mls<sup>c</sup> products, reflecting the overall expression of V $\beta$ 3 in the T cell repertoire. Moreover, they demonstrate the importance of these products in selection of the antigen-specific T cell repertoire.

## Project Description

Major Findings:

1) Series of T cell clones were generated which are specific for Mls<sup>a</sup>, Mls<sup>c</sup>, or Mls<sup>d</sup>. The Mls<sup>a</sup> and Mls<sup>c</sup> specificities of these clones were confirmed by use of panels of inbred strains, recombinant inbred strains, and formal segregation analysis in which the responses of clones were compared with the primary (definitive) T cell responses to MHC identical cells. This analysis demonstrated that Mls<sup>a</sup> and Mls<sup>c</sup> were noncrossreactive as defined by panels of T cell clones, and therefore that the Mls system as defined by panels of T cell clones, and therefore that the Mls system as defined is indeed polymorphic. In addition, it was demonstrated that Mls<sup>d</sup> cells express both Mls<sup>a</sup> and Mls<sup>c</sup> determinants.

2) These studies suggested the possibility that Mls<sup>d</sup> might in fact represent the co-expression of Mls<sup>a</sup> and Mls<sup>c</sup> determinants, a possibility which would imply that Mls<sup>a</sup> and Mls<sup>c</sup> were encoded by non-allelic genes. In order to test this possibility, a formal segregation analysis was carried out in which (Mls<sup>a</sup> x Mls<sup>c</sup>)F<sub>1</sub> x Mls<sup>b</sup> progeny were typed in primary MLR as well as in responses of Mls<sup>a</sup> specific and Mls<sup>c</sup> specific T cell clones. The results of these studies demonstrated conclusively that the genes encoding Mls<sup>a</sup> and Mls<sup>c</sup> determinants are non-allelic and in fact are unlinked. Additional analysis of the progeny from a (Mls<sup>d</sup> x Mls<sup>b</sup>)F<sub>1</sub> x Mls<sup>b</sup> breeding indicated that Mls<sup>d</sup> strains express the products of unlinked genes which are respectively Mls<sup>a</sup>-like and Mls<sup>c</sup>-like. Thus the original concept of Mls as a single locus, multi-allelic polymorphic system is not sufficient to explain these findings. A reappraisal of these results suggests the novel concept that Mls is in fact composed of the products of at least two non-allelic and unlinked genes, with no definitive evidence at present for polymorphism at either of these loci.

3) The influence of MHC gene products in the recognition of Mls was evaluated. It was found that Mls<sup>c</sup> is highly stimulatory to T cells when it is presented on stimulatory cells which also express H-2<sup>k</sup> but not H-2<sup>b</sup> gene products. Experiments employing intra-H-2 recombinant strains demonstrated the importance of I-E region gene products in the phenomenon. Studies employing monoclonal anti-I-A and I-E antibodies also confirmed the importance of these class II molecules in recognition of Mls<sup>c</sup> determinants. A consequence of this MHC influence on Mls stimulatory ability was the re-typing of Mls genotype in a number of strains. The expression of Mls<sup>c</sup> determinants was not previously identified in these strains due to their failure to express a permissive MHC type.

4) The expression of T cell receptor (TCR) V $\alpha$  and V $\beta$  chains was assessed in Mlsc-specific T cell clones. These included clones which were generated by repeated stimulation with Mlsc determinants and clones specific for the antigen pigeon cytochrome c which were crossreactive with Mlsc. V $\beta$ 3 was expressed in 12 of the 13 Mls<sup>c</sup> specific clones analyzed. Although V $\beta$ 11 usage was predominant in



the cytochrome c reactive T cells, it was not expressed in other Mls<sup>c</sup> specific T cells. The striking association suggests that expression of V $\beta$ 3 products alone, independent of other  $\alpha$  or  $\beta$  chain polymorphisms, is sufficient to determine T cell specificity for Mls<sup>c</sup>. It was also observed that strains which themselves express Mls<sup>c</sup>, whether homozygous or heterozygous, delete the expression of V $\beta$ 3 from their peripheral T cell repertoires. This finding is consistent with the interpretation that maintenance of self tolerance to Mls<sup>c</sup> determinants leads to deletion of V $\beta$ 3 expressing T cells.

#### Proposed Course of Research:

Antibodies specific for T cell receptor structures will be utilized to determine whether the receptors used in recognition of Mls<sup>a</sup> and Mls<sup>c</sup> are the same or different from those used in recognition of other specificities. Attempts will be made to establish monoclonal cell lines expressing Mls<sup>a</sup> 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. The observed deletion of V $\beta$ 3 expressing T cells from Mls<sup>c</sup> animals will be employed as a system in which to study the process of self-non-self discrimination. The deletions of V $\beta$ 3 expressing T cells will be studied in thymic and bone marrow chimeras.

#### Publications:

- Abe R, Hodes RJ. Genetic analysis of serologically undefined determinants: A T cell "clonological" analysis of the Mls system. *Current Topics of Microbiology and Immunology* 1988;137:177-182.
- Abe R, Hodes RJ. A reappraisal of Mls genetics. *J Immunogenetics* 1988;15:5-10.
- Abe R, Hodes RJ. Mls determinants recognized by T cells. *J Immunogenetics* 1988;15:11-19.
- Seldin MF, Abe R, Steinberg AD, Hodes RJ, Morse HC. Genetic relationships of Mls<sup>a</sup> among polymorphic loci on distal mouse chromosome 1. *J Immunogenetics* 1988;15:59-66.
- Abe R, Hodes RJ. T cell recognition of Mls<sup>c</sup>. I. Influence of MHC gene products in Mls<sup>c</sup>-specific T cell recognition. *J Immunol.* 1988;140:4132-4138.
- Abe R, Hodes RJ. The Mls system. Non-MHC genes which encode strong T cell stimulatory determinants. *Immunology Today* 1988;9:230-235.
- Abe R, Hodes RJ. The expression of Mls<sup>c</sup> determinants of Mls<sup>a</sup>, Mls<sup>b</sup>, and Mls<sup>x</sup> prototypic strains. *Immunogenetics* 1988;28:221-232.

Needleman BW, Lynch DH, and Hodes RJ. Effect of Mls<sup>a</sup> on antigen presentation to class II-restricted T cells. *J. Immunol.* 1988;141:3760-3767.

Abe R, Vacchio MS, Fox B, and Hodes RJ. Preferential expression of the T cell receptor V $\beta$ 3 gene by Mls<sup>c</sup> reactive T cells. *Nature* 1988;335:827-830.

Abe R, Hodes RJ. Properties of the Mls system: A revised formulation of Mls genetics and an analysis of T cell recognition of Mls determinants. *Immunological Reviews* 1989;107:5-28.

Abe R and Hodes RJ. T cell recognition of minor lymphocyte stimulating (Mls)gene products. *Ann. Rev. Immunol.* 1989;7:683-708.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09266-07 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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 Senior Investigator EIB, NCI

Others: K. Hathcock Chemist EIB, NCI  
 R. Guy Visiting Fellow EIB, NCI  
 P. Henkart Senior Investigator EIB, NCI  
 S. Ullrich Investigator LCB, NCI  
 E. Appella Senior Investigator LCB, NCI

## COOPERATING UNITS (if any)

Department of Immunology, University of Tokyo, Tokyo, Japan

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOXES)

- (a) Human 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 role of both specific and non specific T cell derived signals was demonstrated for activation of B cell antibody responses. It was shown that the supernatants of activated cloned T helper cells are capable of replacing these T cells in the antigen specific and MHC restricted induction of B cell IgG antibody responses. Two distinct and synergizing activities were identified in this supernatant: Interleukin 4, and an antigen-specific and MHC-restricted soluble factor. It was demonstrated that this latter factor can be affinity purified employing monoclonal antibodies specific for T cell receptor V $\beta$ 8 determinants.

Furthermore, metabolic labeling and immunoprecipitation with the same anti-V $\beta$ 8 antibody allowed detection in cell-free helper clone supernatants of a disulfide-linked dimer which is indistinguishable from the T cell-surface  $\alpha\beta$  heterodimer.

A mechanism for the rapid and directed secretion of mediators of T help was demonstrated for the analysis of granule exocytosis by T helper cells. Cloned helper cells, in response to T cell receptor mediated stimuli but not in response to IL2, rapidly secrete the contents of cytoplasmic granules. This provides a potential mechanism for the transmission of B cell activating stimuli from helper T cells to target B cells.

## Project Description

Major Findings:

## 1. Mechanisms of T helper function.

The nature of the signals transmitted by T helper cells to responding B cells in the course of MHC restricted and antigen specific pathways of B cell activation was studied. Cloned helper T cells were activated in vitro either through exposure to specific antigen and antigen presenting cells or through stimulation with solid phase immobilized antibody to the T cell receptor T3 complex. The cell free supernatant generated under these conditions was capable of functioning in an antigen specific and MHC restricted fashion to activate B cell IgG antibody responses. The helper activity of these supernatants was inhibited by monoclonal antibodies specific for IL4, demonstrating that this lymphokine is one of the essential and synergizing components of the helper cell supernatant. A second component, with the properties of both antigen specificity and MHC restriction, was affinity purified using monoclonal antibodies specific for the V $\beta$ 8 determinant of the T cell receptor. These findings demonstrate that both specific and non specific T cell products are capable of synergizing in the activation of B cells. Moreover, they strongly suggest that a cell-free form of the T cell receptor is capable of binding to its specific ligand and mediating T helper cell function.

In order to analyze the cell-free products of cloned T cells at a biochemical level, cells were <sup>35</sup>S-methionine labeled and their supernatant products then evaluated by immunoprecipitation. Monoclonal antibodies specific for constant of variable (V $\beta$ 8-encoded) determinants on the T cell surface  $\alpha\beta$  receptor precipitated material from cell-free supernatants which had a molecular weight of 85-90 kd nonreduced and 40-45 kd under reducing conditions. On reduced-nonreduced 2-dimensional gel analysis, this material was indistinguishable from the cell surface  $\alpha\beta$  heterodimer.

## 2. Granule exocytosis by T helper cells.

In a separate but related set of studies, it was demonstrated that cloned T helper cells bear cytoplasmic granules which contain a series of detectable enzymatic activities. When stimulated with specific antigen or with antibodies directed at T cell receptor structures, but not when activated with IL2, these cloned T helper cells undergo a rapid granule exocytosis. This secretory process provides a mechanism whereby prestored granules containing potentially relevant biologic mediators may play a role in the T helper cell function.



Proposed Course of Project:

The signals involved in T cell-mediated activation of B cell responses will be studied through the characterization of cell free T helper products. The antigen specific T helper factor will be assessed by batch affinity purification. Attempts will then be made to more extensively biochemically characterize this product and in particular to establish its relationship to the cell surface T cell receptor. The ability to activate B cells in a highly specific fashion in the absence of intact T cells will also be employed to study the nature of signal transduction by B cells. Early response parameters such as PI hydrolysis and calcium flux will be studied in B cells activated either through lymphokine, antigen specific soluble helper factor, or combinations of such stimuli.

Publications:

Guy R, Ullrich SJ, Foo-Philips M, Hathcock KS, Appella E, and Hodes RJ. Antigen-specific helper function of cell-free T cell products bearing TCR  $V_{\beta}8$  determinants. Science 1989; in press.

Guy R, Foo-Philips M, and Hodes RJ. Expression of T cell receptor  $V_{\beta}8$  determinants on antigen-specific T helper factor. In Immune System and Cancer, Japan Scientific Societies Press, Tokyo, 1989; in press.

Hodes RJ. T helper-B cell interaction. The roles of direct Th-B cell contact and cell-free mediators. Seminars in Immunology, 1989; in press.

Hodes RJ. T cell-mediated regulation: help and suppression. In Paul, W.E. (Ed.): Fundamental Immunology, 2nd edition, New York, NY, Raven Press, 1989; in press.

Taplits MS, and Hodes RJ. The helper T lymphocyte as a secretory cell: Constitutive versus regulated secretion. In Cruse, J.M. and Lewis, R.E., Jr. (Eds.): The Year in Immunology 1988, Karger, Basel, 1989 pp.147-158.

Taplits, MS, Henkart PA, and Hodes RJ. T helper cell cytoplasmic granules: exocytosis in response to activation via the T cell receptor. J. Immunol. 1988;141:1-9.

Guy R and Hodes RJ. Antigen-specific, MHC restricted B cell activation by cell-free Th2 cell products: Synergy between antigen-specific helper factors and IL4. J. Immunol. 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09267-07 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

T Cell Immune Function in Asymptomatic HIV Seropositive Patients

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

PI:	G. M. Shearer	Senior Investigator	EIB, NCI
	M. Clerici	Visiting Fellow	EIB, NCI
Others:	C. S. Via	Medical Staff Fellow	EIB, NCI
	M. Martin	Senior Investigator	LMM, NIAID
	F. Hakim	Senior Staff Fellow	EIB, NCI
	J. Berzofsky	Senior Investigator	MB, NCI

## COOPERATING UNITS (if any)

R. A. Zajac and R. N. Boswell, HIV Unit, Lackland AFB, TX  
 A. Landay and H. Kessler, Rush Medical Center, Chicago, IL

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

1.7

## OTHER:

0.8

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

Peripheral blood leukocytes (PBL) from HTLV-III antibody (+) and (-) donors were tested for IL-2 production and cell-mediated lympholysis (CML) to recall antigens (S+X) and to HLA allogeneic cells (ALLO). Among antibody (+) donors, approximately 50% failed to respond to S+X, whereas all responded to ALLO, except patients in the critical stages of AIDS, who responded to neither type of immunogen.

Using PBL from antibody (-) donors we demonstrated by cell fractionation techniques that S+X T cell responses are obliged to use CD4<sup>+</sup> T cells, whereas ALLO responses can be generated by either CD4<sup>+</sup> or CD4<sup>-</sup> T cells. Thus, the selective loss of S+X responses probably reflects the loss of CD4<sup>+</sup> cells during AIDS development.

By studying the T helper cell responses in more than 300 HIV<sup>+</sup> patients without AIDS, we have been able to subdivide Walter Reed Stage 1 patients into four subgroups: those that have lost no T helper cell (T<sub>H</sub>) function; those that have lost only T<sub>H</sub> to S+X; those that have lost T<sub>H</sub> function for both S+X and ALLO; and those who have lost T<sub>H</sub> to all antigens and to PHA. We have not seen evidence for a defect in antigen-presenting cell function.

## Project Description

Major Findings:

This laboratory has been investigating T helper cell (TH) immune function and antigen-presenting cell (APC) function in asymptomatic, HIV<sup>+</sup> individuals and patients with symptomatic AIDS. Thus far, we have studied more than 400 subjects. Our studies indicate that four distinct patterns of T helper cell function can be identified within a population of individuals that clinical evaluation would classify as asymptomatic and indistinguishable. These categories include patients with: a) no immune dysfunction; b) a selective defect in response to recall antigens only; c) a defect in response to recall and HLA alloantigens, but not to the nitogen, PHA; and d) a defect in response to all stimuli. Our longitudinal data indicate that there is a progression from a) to d) that is correlated with progression toward AIDS. We have determined that the early selective defect in response to recall but not alloantigens is due to the fact that different TH-APC pathways are utilized in response to these two types of antigens.

We have recently detected APC defects in a subpopulation of AIDS patients, but not in asymptomatic, HIV<sup>+</sup> individuals. This indicates that part of immune deficiency in these patients could be due to a deficiency in antigen-presenting function.

Our study also indicates that a subpopulation of AIDS patients retain intact CD8<sup>+</sup> T cell function, including ability to produce IL-2 in response to HLA alloantigens (ALLO). This finding raises the possibility that alloantigen-stimulated CD8<sup>+</sup> T helpers could provide help for CTL precursors that normally require CD4<sup>+</sup> T helpers. To test this possibility, PBL from HIV<sup>+</sup> individuals who were able to generate CTL to ALLO but not to FLU were cocultured with FLU plus ALLO. In the presence of costimulation with ALLO, the CD4-defective PBL generated CTL responses to FLU. This observation raises the possibility that AIDS patients who have lost CD4<sup>+</sup> T helper cell function but who have retained CD8<sup>+</sup> T helper function and effector function could be immunized in vivo with allogeneic leukocytes plus antigenic determinants against opportunistic infectious agents to which AIDS patients are so susceptible, and possibly to HIV itself. In fact, we have recently seen that PBL from a patient who lost the ability to generate CTL to HIV, were able to generate HIV-specific CTL upon costimulation with ALLO + HIV peptides. This approach offers advantages over the injection of exogenous IL-2 because it should not have the adverse side as other lymphokines that might be important for the activation of T effector cells and B cells.

In collaboration with Dr. Jay Berzofsky (NCI, NIH), we have tested PBL from approximately 150 HIV<sup>+</sup> individuals for proliferative responses IL-2 production and CTL when stimulated with synthetic peptides from the glp20 envelope protein of HIV. We have also tested the proliferative responses of blood from healthy volunteers who were immunized to a vaccinia recombinant that expressed HIV antigens. We found that PBL from the latter group of donors proliferated to the peptides. We found that PBL from approximately 30% of the infected donors did not proliferate to the peptides, but did produce IL-2 when stimulated with the

peptides. Recent collaborative studies with Dr. M. Martin (NIAID) indicated that infant mice that express the human HIV gene do not exhibit changes in CD4<sup>+</sup> or CD8<sup>+</sup> T cell numbers, but do show elevated numbers of B cells. Spleen and lymph node cells from these mice exhibit defects in T lymphocyte function.

Publications:

Shearer GM, Singer A, Buller RM, Ambrus H, Morse, HC. Importance of CD8<sup>+</sup> T helper cell function in AIDS (Letter to the Editor) J. Infect. Dis. 1988; 158:893.

Berzofsky JA, Bensussan A, Cease KB, Bourge JF, Cheynier R, Zirimwabagangabo L, Salaun J-J, Gallo RC, Shearer GM, Zagury D. Antigenic peptides recognized by T lymphocytes from AIDS viral envelope-immune humans. Nature 1988;706-708.

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Leonard JM, Abramczuk JW, Penzen DS, Rutledge R, Belcher JH, Hakim F, Shearer, G, Lamperth L, Travis W, Fredrickson T, Notlzins AL, and Martin, MA. Development of disease and virus recovery in transgenic mice containing HIV proviral DNA. Science 1988;242:1665-1670.

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Morse HC III, Yetter RA, Via CS, Hardy RR, Cerny A, Hawakawa K, Hugin A, Miller MW, Shearer GM. Functional and phynotypic alterations in T cell subsets during the cause of MAIDS, a murine retrovirus-induced immunodeficiency syndrome. J. Immunol. 1989; in press.

Clerici M, Stocks NI, Zajac RA, Boswell RN, Lucey DR, Via CS and Shearer GM. Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, HIV-seropositive patients: Independence of CD4<sup>+</sup> cell numbers and clinical staging. J. Clin. Invest. 1989; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09268-02 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Role of CD4 and CD8 Accessory Molecules in T Cell Function

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

PI:	A. Singer	Senior Investigator	EIB, NCI
Others:	S. McCarthy.	Senior Staff Fellow	EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.25

## PROFESSIONAL:

1.25

## 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.)

We have examined the role of CD4 and CD8 in MHC class recognition. In these studies, we have evaluated the ability of CD4 and CD8 molecules to function as signalling molecules for mature and developing T cells. Interestingly, we have found that CD4 function is related to TcR expression and appears to play an important signalling role in T cell differentiation. Indeed, there are a number of indications in these studies that CD4 and CD8 can both function as signalling molecules, but that they are not functionally identical molecules that merely differ in their ligand specificity for MHC class I or MHC class II determinants.

## Project Description

Major Findings:

The concept that MHC class specificity expressed by T cells is determined by their cell surface expression of either CD4 or CD8 accessory molecules implicitly presumes that CD4 and CD8 molecules are functionally equivalent receptor molecules that differ only in their ligand specificity. Since activation of protein kinase C (PKC) by phorbol esters such as PMA can mimic receptor-mediated signalling and cause phosphorylation, internalization, and down-regulation of surface receptors, we examined if the responses of CD4 and CD8 to PKC activation were identical. To our surprise, we found that even though PMA activation of PKC induced rapid internalization and decreased cell surface expression of TcR on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, the responses of the CD4 and CD8 molecules themselves to PKC activation were markedly divergent. For example, PMA induced PKC activation in CD4<sup>+</sup>CD8<sup>+</sup> "double positive" thymocytes revealed, in a single cell population, a marked and rapid decrease in cell surface expression of CD4 but nearly unchanged cell surface expression of CD8 over 5 hours. Thus, CD4 behaved similarly to molecules comprising the TcR complex in response to PKC activation, whereas CD8 did not. The relationship between CD4 and TcR was further examined by assessing the effect of anti-CD4 mAb on TcR expression on mature T cells. We found that anti-CD4 mAb caused the internalization of CD4 and the co-internalization of TcR. PKC or a related cellular intermediary was involved in these co-internalization events, since PKC depleted T cells were unable to co-internalize TcR in response to anti-CD4 mAb.

Because CD8<sup>+</sup> anti-class II T cells constitute the only "aberrant" T cell subset whose phenotype is "discordant" with its MHC specificity, we wished to compare their MHC differentiation requirements with those of "conventional" CD4<sup>+</sup> anti-class II T cells whose phenotype was "concordant" with their MHC specificity. Differentiation of all CD4<sup>+</sup> T cells, including CD4<sup>+</sup> anti-class II T cells, requires engagement of thymic MHC class II determinants as indicated by the arrest of CD4<sup>+</sup> T cell differentiation by in vivo administration of saturating amounts of anti-Ia antibodies. Consequently, we examined if differentiation of class II specific CD8<sup>+</sup> T cells was similarly arrested by in vivo anti-Ia blockade. Interestingly, we found that anti-Ia blockade did not interfere with the differentiation of CD8<sup>+</sup> anti-class II T cells even though, in the same animals, it arrested the differentiation of CD4<sup>+</sup> anti-class II T cells. These results demonstrated that differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> anti-class II T cells differed fundamentally in their requirements for Ia engagement. Indeed, these results indicated that differentiation of CD8<sup>+</sup> T cells, even those whose receptors had specificity for MHC class II determinants, did not depend on engagement of thymic class II determinants, but, instead, either did not require MHC engagement at all or required engagement of MHC class I determinants. These observations have suggested to us that the MHC requirements for differentiation that developing T cells express may not reflect a requirement for TcR engagement, but rather may reflect a requirement for MHC-induced cross-linking of CD4 or CD8 accessory molecules.

To examine the effect of CD4 cross-linking on developing T cells, we injected neonates with anti-CD4 mAb and examined TcR expression on the developing thymocytes. Remarkably, we found that the mAb-induced crosslinking of CD4 on CD4<sup>+</sup>8<sup>+</sup> double positive thymocytes caused a 3-5 fold increase in their surface expression of TcR. This was a rapid effect, occurring within 16h, and was a direct effect on the immature double positive thymocytes themselves. Interestingly, the effect of anti-CD4 engagement on the mature single positive thymocytes within the same thymus was to reduce TcR expression. Thus, the effect of anti-CD4 engagement on TcR expression was a function of the developmental state of the responding T cell.

We examined the role of CD8 in T cell function by assessing the function of CD8 on precursor Tk (pTk) cells that would differentiate, upon activation, into mature Tk cells. Since anti-CD8 mAbs blocks recognition of class I target cells by most mature CD8<sup>+</sup> anti-class I Tk cells, it seemed reasonable to expect that anti-CD8 mAbs would similarly block CD8<sup>+</sup> pTk cell recognition of class I stimulator cells in the induction cultures and so inhibit anti-class I Tk cell responses. However, this turned out not to be the case. We found that addition of anti-CD8 mAbs to the induction cultures did not block generation of anti-class I Tk cells responses, but, paradoxically, frequently enhanced them. However, the anti-class I Tk cells that were generated in the presence of anti-CD8 mAbs were different than the anti-class I Tk cells that were generated in conventional induction cultures devoid of anti-CD8 mAb. Conventional Tk cells were overwhelmingly anti-CD8-sensitive in their ability to lyse class I targets, whereas novel Tk cells from cultures containing anti-CD8 mAbs were overwhelmingly anti-CD8-resistant. We found that the underlying mechanism responsible for anti-CD8 mAb induction of anti-CD8-resistant Tk cells involved: (a) multivalent cross-linking of CD8 molecules on the pTk cells, and (b) TcR engagement. We found that bivalent cross-linking of CD8 molecules on pTk cells did not lead to internalization and downmodulation of cell surface CD8, and resulted in blockade of pTk cell activation. In contrast, we found that multivalent cross-linking of CD8 molecules on pTk cells did lead to downmodulation of cell surface CD8 and did activate pTk cells to differentiate into anti-CD8-resistant Tk cells. Thus, these studies functionally demonstrate a signalling role for CD8 in Tk cell activation that requires multivalent cross-linking of CD8 to other CD8 molecules. In the light of these results, it is possible that activation of CD8<sup>+</sup> T cells requires two independent signals: one induced by CD8 cross-linking and one induced by TcR cross-linking.

#### Publications:

Mizuochi T, Tentori L, Sharrow SO, Kruisbeek AM, and Singer A. Differentiation of Ia-reactive CD8<sup>+</sup> murine T cells does not require Ia engagement. Implications for the role of CD4 and CD8 accessory molecules in T cell differentiation. *J. Exp. Med.* 1988;168: 437-442.

Kaldjian E, McCarthy SA, Sharrow SO, Littman DR, Klausner RD, and Singer A. Nonequivalent effects of PKC activation by PMA on murine CD4 and CD8 cell surface expression. *FASEB J.* 1988;2:2801-2806.

McCarthy SA, Kruisbeek AM, Uppenkamp IK, Sharrow SO, and Singer A. Engagement of the CD4 molecule influences cell surface expression of the T-cell receptor on thymocytes. *Nature* 1988;336:76-79.

McCarthy SA, Kaldjian E and Singer A. Induction of anti-CD8 resistant CTL by anti-CD8 antibodies: Functional evidence for T cell signalling induced by multivalent cross-linking of CD8 on precursor cells. *J. Immunol.* 1988;141:3737-3745.

McCarthy SA, Kaldjian E and Singer A. Multivalent cross-linking of CD8 transduces an activation signal in developing cytolytic T lymphocytes. In Kaplan JG, Green DR and Bleackley RC (Eds.): *Cellular Basis of Immune Modulation*. Alan R. Liss, New York, NY, 1989;pp 113-116.

Zunia-Pflucker JC, McCarthy SA, Weston M, Longo DL, Singer A, and Kruisbeek AM. Role of CD4 in thymocyte selection and maturation. *J. Exp. Med.* In press.

Cole JA, McCarthy SA, Rees MA, Sharrow SO and Singer A. Cell surface co-modulation of T cell receptor by anti-CD4 monoclonal antibody. *J. Immunol.* In press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09269-06 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Sequence Organization of Class I MHC Genes

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

PI: Dinah Singer Senior Investigator EIB, NCI

Others: Stuart Rudikoff Senior Investigator LG, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

3.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The aim of this work is to determine the DNA sequence organization of class I genes contained in the swine major histocompatibility complex (SLA). We have established that this gene family contains only seven members, of which five have been isolated and their DNA sequences determined. It has been established that the sequence organization of the class I SLA genes is similar to that of other class I genes in other species. Within the family, it is possible to define at least three sub-families, based on their sequences. Homologies between the SLA genes range from 85% between two genes within a sub-family to 45% between sub-families. Gene conversion events appear to give rise to complex alterations, and to occur between sub-families. Physical mapping has established that the class I genes map within 500 kb on a single linkage group.

Isolation of cDNA clones has demonstrated that only 4 class I genes are expressed within swine PBL populations. Sequence analysis of these clones has established the locations of exons and further indicates a variety of post-transcriptional alternative splicing events occurs in these cells.

## Project Description

Major Findings:

We have determined the DNA sequences of the five isolated class I SLA genes. From their sequence homologies, we have identified three sub-families. One sub-family consists of PD1, PD14 and PD7, which are between 81-85% homologous to one another. The other two sub-families contain only a single member. These genes, PD6 and PD15, are very divergent both from the PD1 sub-family (49-55%) and from each other (45%). By comparison with class I genes from other species, all of the members of the PD1 sub-family are composed of eight exons; in contrast, PD6 has only seven exons and PD15 has no discernible exons 6-8. In order to isolate the remaining two class I genes, we have screened a cosmid library, and are currently characterizing the isolated clones.

The physical linkage map of the class I locus is being investigated in two ways. Large scale mapping of the pig genome by pulsed field electrophoresis of DNA digests generated by restriction enzymes which cut infrequently within the genome shows that the SLA class I complex is contained within a single 500 kb segment, considerably smaller than the HLA class I complex, which has been estimated to span 2000 kb. To order individual genes more precisely cosmid clones have been isolated. Of the clones isolated, many have two or more genes contained within them within 20 kb of each other. Thus, the spacing of class I SLA genes is similar to that of HLA genes. By combining the data obtained from the two approaches, it will be possible to generate a fairly accurate map of the gene family without necessitating extensive cosmid walking. Two of the genes isolated from cosmids have been sequenced.

Because the splicing patterns of the SLA class I genes and their corresponding intron-exon boundaries had been inferred by comparison with class I genes of man and mouse, we isolated cDNA clones for the purpose of: 1) determining which of the seven genes could be expressed *in vivo*, and 2) the splicing patterns and 5' and 3' untranslated sequences. To this end, a cDNA library was constructed from RNA of peripheral blood lymphocytes and screened with both PD1 and PD6 probes. cDNA clones were isolated and grouped according to their hybridization pattern and restriction maps. Examples of each group were sequenced, yielding the cDNA sequences of PD1, PD14, PD7 and PD6. These cDNA sequences have confirmed our assignments of exon/intron junctions and have identified the sites of transcriptional initiation and termination.

Publications:

Singer D, Ehrlich R, Maguire J, Golding H, Satz M, Parent L, and Rudikoff S. Structure and organization of class I MHC genes in miniature swine. *Molecular Biology of the MHC of Domestic Animal Species*. Editors: C. Warner, M. Rothschild, S. Lamont, Iowa State University Press, Ames, Iowa, 1988.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09270-06 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Regulation of Expression of Class I MHC Genes

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

PI: Dinah Singer Senior Investigator EIB, NCI

Others: Jean Maguire Biotechnology Fellow EIB, NCI  
 Jocelyn Weissman Chemist EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

7.0

## PROFESSIONAL:

7.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The aim of this work is to investigate the mechanisms controlling the expression of a multi-gene family. The miniature swine has been chosen as an experimental model because there are only 7 members of the family. To address the question of the molecular regulation of the expression of class I MHC genes, two approaches have been taken: 1) the analysis of in vivo patterns of expression of each of the genes, both in situ and in transgenic mice; and 2) characterization of regulatory elements associated with these genes.

Analysis of in vivo patterns reveals that both shared and discrete regulatory mechanisms operate within the family. Thus, some genes are coordinately expressed, whereas others are not. In both cases, patterns of expression are actively regulated.

Regulatory sequences within one of the transplantation antigen genes have been identified by generating a series of 5' end deletion mutants. A series of both positive and negative elements have been functionally mapped by transfection into cell lines. In vivo function of identified elements has been examined by generating transgenic mice containing relevant deletion mutants. A novel negative regulatory element, which requires the presence of a positive element in order to function, has been identified. It has been further demonstrated that these elements function through the binding of trans acting factors. The binding sites of the trans acting factors have been mapped using both gel retardation assays and exonuclease protection.

## Project Description

Major Findings:

Expression of individual class I genes is actively regulated: multiple genes within the family show similar patterns and responses, whereas other class I antigens, such as Qa and TL, have been classified as differentiation antigens because of their limited tissue distribution. We speculate that common mechanisms exist which coordinately regulate certain class I gene expression and discrete mechanisms exist which regulate expression of the differentiation antigens. In earlier studies, we demonstrated that introduction of one of the swine class I genes, PDI, into mouse L cells resulted in its regulated expression. Furthermore, interferon treatment of transfected L cells increased transcription of the PDI gene, indicating that regulatory sequences were contained within the transfected DNA segment. In order to further define the regulation of PDI, we have examined its *in situ* patterns of expression as well as its expression in transgenic mice. In addition, we have constructed a variety of deletion mutants generated from the 5' flanking sequences which have allowed us to identify and define a series of regulatory elements. We are presently conducting parallel experiments with other members of this family in order to compare their regulation.

We have undertaken a molecular dissection of PDI with the aim of identifying functional regulatory DNA sequence elements. To date, our studies have focussed primarily on the 1.1 Kb of 5' flanking region contained within the transgene. Using a limited series of 5' deletion mutants, ligated to the reporter gene CAT, we have identified the transcriptional promoter, the interferon response element (IRE) and an array of positive and negative regulatory elements. Of particular interest is the existence of a novel negative element. Removal of this element from the 5' flanking segment increases expression from the PDI promoter by 5-10 fold. Introduction of the silencer segment adjacent to the heterologous viral SV40 promoter similarly reduces its efficiency of transcription. However, this negative effect is observed only if the SV40 promoter has its enhancer element associated with it. In the absence of the viral enhancer, the PDI silencer does not function. *In vivo* competition experiments, employing various combinations of enhancers and the PDI silencer have demonstrated that trans acting factors mediate the function of these elements. In particular, a PDI enhancer shares at least some common elements with the SV40 enhancer. Furthermore, the trans acting factor(s) binding to the silencer element does so only in the presence of an enhancer and its cognate binding factors. Although the molecular mechanisms by which the regulatory elements and their factors function is not known, our data suggest a model in which the negative and positive regulatory elements each define an independent binding site .

Functional analysis of the PDI 5' flanking region has documented the existence of at least three enhancers and a silencer (see above). An extended series of 5' deletion mutants has generated a fine map of the position and function of these elements. In order to analyze the *in vivo* function of these elements, transgenic mice are being generated with various of these constructs. Tissue patterns of expression of these transgenic animals are being compared to assess the roles of the DNA elements in regulating class I gene expression.



Inspection of the DNA sequence of the PD1 5' flanking region revealed no striking homologies with known regulatory elements from other gene systems, other than the interferon responsive element, the H2TF1 binding site, and enhancer B. However, a 140 bp DNA sequence in this region contains an inverted repeat which overlaps with a region of dyad symmetry. Functional analyses have mapped enhancer and silencer function to these elements. Further, distinct trans acting factors are associated with these elements. These have been identified by gel mobility shift assays and foot-printing techniques. Studies are underway to isolate these factors.

The patterns of expression of four other class I genes have been examined. One, PD14, encodes a classical transplantation antigen and is coordinately expressed with PD1. A second gene, PD7, has been shown to have precisely the same pattern of expression as PD1, but at markedly reduced RNA levels relative to PD1 in all tissues. Transfection of PD7 into mouse L cells results in a very low level of transcription and commensurately low level of cell surface expression. Removal of 3 kb of 5' flanking sequence from PD7 increases its level of transcription. A third gene, PD6, displays patterns of expression distinct from PD1. Although PD6, like PD1, is expressed in pigs at the highest levels in lymphoid tissues, unlike PD1, it is preferentially expressed in T cells, relative to B cells, and is not expressed at all in the thymus. The fourth gene, PD15, is not detectably expressed in any adult tissue nor in transfected L cells, and is considered to be a pseudogene.

#### Publications:

Singer DS and Ehrlich R. Identification of regulatory elements associated with a class I MHC gene. *Current Topics in Microbiology* 1988;137:148-154.

Singer DS, Ehrlich R, Maguire J, Golding H, Satz L, Parent L, and Rudikoff S. Structure and organization of class I MHC genes in miniature swine. *Molecular Biology of the MHC of Domestic Animal Species*, Editors: C. Warner, M. Rothschild, S. Lamont. Iowa State University Press, Ames, Iowa, 1988.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09271-06 E

PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Graft-versus-Host Disease in Allogeneic Marrow Transplantation and Tumor Purging		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	R. E. Gress	Senior Investigator EIB, NCI
Others:	R. Moses	Biotechnology Fellow EIB, NCI
	R. Hirsh	Medical Staff Fellow EIB, NCI
	H. Nakamura	Visiting Fellow EIB, NCI
COOPERATING UNITS (if any)		
LAB/BRANCH Experimental Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	
CHECK APPROPRIATE BOX(ES)		
<input checked="" type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
		A
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Graft-versus-host disease (GvHD) is dependent in its generation on the presence of immune competent T cells in the donor marrow with specificity for allogeneic antigens of the host. The activation of these cells in the host after infusion and the resultant tissue injury is clinically recognized as GvHD. One approach to preventing GvHD is the removal of these T cells from the marrow inoculum. We have pursued T cell depletion of human marrow for the purpose of preventing GvHD in marrow transplantation across MHC differences in man by the use of monoclonal antibodies specific for cell surface molecules unique to T cells in combination with complement. Two such antibodies have been derived, one with specificity for CD2 (sheep red blood cell receptor) and one which defines a novel T cell determinant. The latter antibody precipitates a 92 KD molecule and a predominant 45 KD band under non-reducing conditions and reducing conditions, respectively. These two antibodies, in combination with CD7 and CD5 specific antibodies, have been used to carry out studies in human bone marrow T cell depletion. To quantitate T cells remaining in marrow after such depletions, a limiting dilution assay was developed which detects T cells at a level of one T cell per <math>10^5</math>-<math>10^6</math> marrow cells. A bank of cryopreserved marrows depleted of T cells by a combination of monoclonal antibodies and complement, with the extent of that depletion assessed by limiting dilution assay, has been established. The techniques developed for the removal of normal T cells from allogeneic marrow have also been applied to the removal of malignant T cells from autologous marrow. A clinical protocol has been developed to assess the feasibility of utilizing these marrows in the treatment of aggressive T cell malignancies with marrow ablative chemotherapy and radiotherapy.</p>		

## Project Description

Major Findings:

The primary approach taken in these studies of T cell depletion of human marrow has been by antibody and complement; alternative approaches such as the use of immunotoxins, elutriation, and immune rosetting have also been evaluated. Initial studies with antibody and complement established conditions for optimal complement mediated lysis and showed that individual antibodies differed in their ability to effect lysis in the presence of complement. A combination of antibodies was superior to single agents not in the extent of depletion, but in reproducibility from treatment to treatment. In addition to two antibodies available from other sources (CD 7 and CD 5 specific), neither of which was adequate to mediate pan T cell depletion effectively, we derived additional monoclonal antibodies with screening by complement mediated lysis. Two antibodies of interest were isolated. One was CD2 specific and has been utilized in these studies for T cell depletion and in investigations of the role of the LFA-2 accessory molecule in T cell function. It detects an epitope region distinct from that detected by OKT11. The second antibody is specific for a novel determinant on T cells. The determinant for which this antibody is specific is expressed by cells of hematopoietic origin, is confined to T cells, and is concordant with the expression of CD5 and CD3. Immunoprecipitation demonstrates a 92 KD molecule under non-reducing conditions and a predominate 45 KD band under reducing conditions. Comparisons of expression on a series of T cell lines, including a line deficient in the expression of T cell receptor, distinguished the determinant defined by this antibody from those detected by known anti-T cell monoclonal antibodies.

The number of donor marrow T cells necessary for the generation of GVHD is on the order of 0.1% in the mouse or  $1 \times 10^5$ /kg in man. Assays commonly used for the quantitation of residual T cells after depletion such as proliferative responses to mitogens (PHA) or alloantigens (MLR) or analysis by FACS detect on the order of 1% T cells and so are insufficient in sensitivity. A limiting dilution assay was therefore developed based on the clonogenic potential of peripheral human T cells. The readout is cell growth; the sensitivity of this assay is sufficient to detect one T cell in  $10^5$ - $10^6$  marrow cells and the specificity has been confirmed by a variety of techniques. This limiting dilution assay has been essential to the monitoring of T cell depletion from human marrow. With this assay, the depletion regimen was refined and different methods of marrow harvest evaluated with respect to influence on final marrow T cell content. Surgical resection of marrow was superior to conventional aspiration as a method of harvest. One consideration in the clinical use of surgically resected marrow is marrow cell yield. The final yield in a series of harvested marrows (after treatment to remove T cells) was 74% of the Ficoll population for a final mean total cell number of  $9.3 \times 10^9$  cells per donor. The residual T cell content was less than one T cell per  $10^5$  marrow cells which represented, in each case, greater than a three log depletion of T cells. Progenitor cell (CFU-C and BFU-E) recovery was 42-100%. These and similarly prepared marrows have been cryopreserved as a "universal donor bank". In evaluating alternative methods of T cell depletion, the most effective and reproducible was elutriation. The processing of human marrow for clinical use

has now been adapted to a semi-automated system, which includes elutriation followed by treatment with antibody plus complement, and has been applied to the removal of malignant T cells from autologous marrow.

Publications:

Quinones R, Lunney J, and Gress, RE. An anti-human T cell monoclonal antibody with specificity for a novel determinant. Transplantation 1988;46:143-150.

Moses RD, Beschorner WE, Singer D, Orr KS, Bacher JD, and Gress, RE. Restriction fragment length polymorphism analysis with a crossreactive HLA class II DR-beta gene probe for the detection of engraftment of MHC-mismatched marrow in the rhesus monkey. Bone Marrow Transplantation; in press.

Gress RE. Bone Marrow Transplantation. Current Opinions in Immunology; in press.

Gress RE, Lucas PJ, Cassel J, Nakamura H, Moses RD, and Quinones RR. Efficacy of T cell depletion of surgically resected cadaveric marrow: Considerations in HLA-mismatched marrow transplantation. In Gress, S. and Gee, A. (eds.): Bone Marrow Purging. Alan R. Liss, Inc.; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09273-02 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

T Cell Differentiation and Repertoire Selection

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

PI:	A. Singer	Senior Investigator	EIB, NCI
Others:	E. W. Shores	Guest Researcher	EIB, NCI
	S. A. McCarthy	Senior Staff Fellow	EIB, NCI
	T. Nakayama	Visiting Fellow	EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.35

## PROFESSIONAL:

1.35

## 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.)

We have examined thymic influence on the MHC-specificity expressed by functionally and phenotypically distinct T cell subsets. Studies on thymocytes from genetically defective scid mice have suggested that TcR( $\gamma\delta$ ) cells may play a critical role in promoting the entry of thymocytes into the CD4/CD8 differentiation pathway. Other studies examining the signalling role of CD4 and CD8 accessory molecules in the selection and differentiation of immature CD4<sup>+</sup>8<sup>+</sup> double positive thymocytes have revealed that these molecules do function as signalling molecules and do influence TcR expression on these immature cells.

## Project Description

Major Findings:

In order to examine the general relationship between TcR expression and T cell differentiation, we have examined a genetically defective mouse strain. Mice with severe combined immune deficiency (scid), lack both receptor bearing T cells and receptor bearing B cells. It is thought that this genetic defect results from a deficiency in the recombinase enzymes necessary for receptor gene rearrangements, making it very difficult for the lymphocytes in these animals to express any antigen receptors. As a result, these animals represent an excellent model for examining how far T cell differentiation can progress in the absence of TcR expression, and for indicating which steps are in fact dependent on TcR. In normal T cell ontogeny, T cells can be characterized by their expression of CD4 and CD8 accessory molecules. Thus, the kinetics of appearance of thymocyte subsets is:  $CD4^-CD8^- \rightarrow CD4^+CD8^+ \rightarrow CD4^+CD8^-$ ,  $CD8^+CD4^-$ . The  $CD4^+CD8^+$  double positive subset consists of approximately equal numbers of  $TcR^+$  and  $TcR^-$  cells, suggesting that TcR expression is not necessary for thymocytes to become  $CD4^+CD8^+$ . Consequently, the expectation was that, in scid mice, thymocyte development would proceed through the generation of double positive cells. To our surprise, we have found that  $Thy1^+$  thymocytes from most scid mice contain only  $CD4^-CD8^-$  (double negative)  $TcR^-$  cells. These cells are  $IL-2R^+$  and  $Lyl$  dull, and so are similar to double negative cells from the thymic of normal mice. What is surprising from these results is that, in the absence of  $TcR^+$  cells, T cell differentiation does not proceed beyond double negative cells. We found that in "leaky" scid mice (those few mice that spontaneously express  $TcR^+$  cells) the intra-thymic presence of  $TcR^+$  cells was invariably accompanied by the intra-thymic presence of  $CD4^+/CD8^+$  cells. Surprisingly, however, the  $CD4^+/CD8^+$  cells were not necessarily themselves  $TcR^+$ . We further examined this apparent paradox by reconstituting irradiated scid mice with bone marrow stem cells from normal AKR mice that would give rise to normal  $TcR^+$  AKR thymocytes. In these thymic, the scid thymocytes remained  $TcR^-$  but became  $CD4^+/CD8^+$ . Thus, intra-thymic  $TcR^+$  cells were able to promote the differentiation of  $TcR^-$  thymocytes into  $CD4/CD8$  expressing cells. When applied to normal T cell differentiation, these results suggest a critical role for early appearing  $TcR(\gamma\delta)$  thymocytes in initiating the entry of  $CD4^-8^-$  double negative thymocytes into the  $CD4/CD8$   $TcR(\alpha\beta)$  differentiation pathway.

If thymic selection occurs at the  $CD4^+8^+$  DP stage of differentiation,  $CD4^+8^+$  DP thymocytes must be able to respond to membrane-initiated signals. To examine this possibility, we examined the ability to activate tyrosine kinase and to phosphorylate the zeta chain of the TcR complex in DP thymocytes. We found that  $CD4$ ,  $CD8$ ,  $CD3$ , and  $TcR(\alpha\beta)$  were able to induce tyrosine phosphorylation of zeta in DP thymocytes. Interestingly, we found that ligand would similarly cause zeta phosphorylation. Thus, these studies demonstrate that DP thymocytes are receptive to membrane signals, and that four of these membrane signalling molecules are  $CD3$ ,  $CD4$ ,  $CD8$ , and  $TcR(\alpha\beta)$ .

To understand the molecular basis for increased TcR expression on DP thymocytes in response to CD4 engagement, we compared anti-CD4 induced and untreated DP thymocytes. We found that anti-CD4 treatment resulted in a 3-5 fold increase in TcR, but that this increase was not due to increased synthesis or assembly. Rather, this increase was due to decreased degradation in DP thymocytes in response to CD4 engagement. Thus, these studies have identified an unexpected mechanism regulating TcR expression during development.

Publications:

Mizuochi T, Tentori L, Sharrow SO, Kruisbeek AM, and Singer A. Differentiation of Ia-reactive CD8<sup>+</sup> murine T cells does not require Ia engagement. Implications for the role of CD4 and CD8 accessory molecules in T cell differentiation. *J. Exp. Med.* 1988;168: 437-442.

McCarthy SA, Kruisbeek AM, Uppenkamp IK, Sharrow SO, and Singer A. Engagement of the CD4 molecule influences cell surface expression of the T-cell receptor on thymocytes. *Nature* 1988;336:76-79.

McCarthy SA and Singer A. T-lymphocyte associative recognition. In Litwin SD, Scott DW, Flaherty L, Reisfeld RA, and Marcus DM (Eds): *Human Immunogenetics: Basic Principles and Clinical Relevance*. Marcel Dekker, Inc. New York, 1989;pp399-442.

Zunia-Pflucker JC, McCarthy SA, Weston M, Longo DL, Singer A, and Kruisbeek AM. Role of CD4 in thymocyte selection and maturation. *J. Exp. Med.*

Owen JJT and Singer A. Workshop summary: Thymocyte differentiation and positive selection. In Janeway CA, Sprent J, and Sercarz E (Eds): *Immunogenicity*. Alan R. Liss, New York, NY.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09275-02 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

In Vivo Study of MHC-Specific T Cells

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

PI: A. Singer Senior Investigator EIB, NCI

Others: M. Rees Guest Researcher EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.25

## PROFESSIONAL:

1.25

## 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 has attempted to apply our understanding of the cellular mechanisms involved in in vitro anti-MHC responses to in vivo transplantation responses. In studying skin allograft rejection, we have identified the phenotype, specificity, and interaction capabilities of the T cells able to initiate and effect in vivo rejection responses. Using allophenic skin grafts, we found that the effector mechanism of skin graft rejection is itself antigen-specific, and that defects in Th cell function result in longterm retention of skin allografts despite the presence of antigen-specific effector cells. In addition, we have assessed the cellular mechanisms mediating the rejection of fetal pancreas and Islet cell allografts.



## Project Description

Major Findings:

We have been studying transplantation immunity as the in vivo analog of the anti-MHC responses we had been examining in vitro. Consequently, our initial focus was on determining the phenotype, function, and specificity of the T cells that initiate and mediate in vivo rejection responses. We chose to first study the rejection of skin allografts because skin allografts trigger the most potent of all in vivo rejection responses, and because they are convenient to assess and follow.

To directly identify the T cell populations involved in skin allograft rejection, we developed an adoptive transfer model in which H-2<sup>D</sup> nude mice were engrafted with allogeneic skin and then reconstituted with T cells or T cell subpopulations. In this experimental model the rejection of skin allografts was shown to depend upon and be mediated by the adoptively transferred T cells. Skin allografts expressing whole MHC disparities were rejected by negatively selected populations of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells. In contrast, class I disparate skin allografts were only rejected by CD8<sup>+</sup> T cells, whereas class II disparate and minor H disparate skin allografts were only rejected by CD4<sup>+</sup> T cells. These results demonstrated that skin allograft rejection was not a function unique to one phenotypic subset of T cells, but could be mediated by either CD4<sup>+</sup> or CD8<sup>+</sup> T cells, depending upon the class of histocompatibility antigens the graft expressed. The functional capabilities expressed in vitro by CD4<sup>+</sup> and CD8<sup>+</sup> T cells responding to a wide variety of different histocompatibility antigens was compared with their ability upon adoptive transfer to reject skin allografts expressing those histocompatibility antigens. These studies revealed that, without exception, skin allografts were only rejected by T cell populations that contained, by in vitro assessment, both lymphokine secreting Th cells and cytolytic Tk cells specific for the antigens of the allograft. Thus, skin allograft rejection responses require the activation of antigen-specific, lymphokine secreting Th cells and the generation of antigen-specific Tk cells. Further studies demonstrated that these two functions could either be mediated by distinct populations of interacting Th and Tk cells, or, alternatively, could be mediated by a single population of dual function T cells that both secrete lymphokines and differentiate into cytolytic effector cells.

These studies strongly supported the concept that in vivo rejection responses, once initiated by antigen-specific Th cells, are mediated by antigen-specific Tk cells. However, they did not directly demonstrate that antigen-specific Tk cells actually mediate the rejection process. Indeed, it was conceivable that allograft rejection is effected by lymphokines secreted by antigen-specific Th cells or DTH cells that either nonspecifically recruit inflammatory effector cells or induce intense vasospasm, causing nonspecific ischemic necrosis of the graft. To distinguish between antigen-specific and antigen-nonspecific effector mechanisms of allograft rejection, we constructed B6<->A/J allophenic mice which

are genetic mosaics whose individual cells express either H-2<sup>b</sup> or H-2<sup>a</sup> determinants, but not both. The fur of these B6<->A/J allophenic mice consists of both black and white hairs, the black hair resulting from pigment donated by B6 (H-2<sup>b</sup>) melanocytes and the white hair resulting from A/J (H-2<sup>a</sup>) melanocytes that are genetically unable to synthesize pigment. Skin from these allophenic mice were grafted onto H-2<sup>b</sup> nude mice and rested for 1 month to permit the grafts to regrow their black and white hairs. Because nude mice are immunoincompetent, the experimental grafts were not rejected. The animals were then reconstituted with H-2<sup>b</sup> T cells responsive against the H-2<sup>a</sup> cellular elements of the allophenic grafts but tolerant to its H-2<sup>b</sup> elements. Within two weeks of the T cell transfer, the grafts were enveloped by an intense non-specific inflammatory response that caused all the hairs of the allophenic grafts to fall out. Nonetheless, the allophenic grafts survived the inflammation, and, most remarkably, regrew hair. The color of the regrown hair was overwhelmingly black, the color of the syngeneic H-2<sup>b</sup> parent, providing visual proof that the effector mechanism of skin allograft rejection destroys cells expressing allogeneic histocompatibility antigens but spares cells that do not. Thus, the effector mechanism of skin allograft rejection is MHC-specific and, therefore, mediated by a T<sub>k</sub> cell that assesses individual cells in the dermis of the graft for expression of foreign histocompatibility antigens.

To examine the general applicability of these conclusions, we have also examined the cellular basis by which fetal pancreas and islet allografts are rejected. We found that the cellular mechanisms involved in the rejection of fetal pancreas allografts were identical to those involved in skin allografts. Furthermore, we found that fetal pancreas allografts did not undergo graft adaptation despite a 9 month residence in an immunoincompetent host. In contrast, we found that rejection of isolated islet cell allografts was solely dependent on CD4<sup>+</sup> T cells. This finding raises a number of issues that will need to be clarified in the future, including how CD4<sup>+</sup> T cells reject cells that lack MHC class II expression, and why CD8<sup>+</sup> T cells are unable to reject Islet cell allografts.

#### Publications:

- Rosenberg AS, Mizuochi T, and Singer A. Evidence for involvement of dual function T cells in rejection of MHC class I disparate skin grafts. Assessment of MHC class I alloantigens as in vivo helper determinants. *J. Exp. Med.* 1988;168:33-46.
- Rosenberg AS, Mizuochi T, and Singer A. Cellular interactions resulting in skin allograft rejection. In Battisto JR, Plate J and Shearer GM. (Eds.): *Cytotoxic T Cells. Biology and Relevance to Disease.* Annals N.Y. Acad. Sci. 1988;532:76-86.
- Rosenberg AS and Singer A. Evidence that the effector mechanism of skin allograft rejection is antigen specific. *Proc. Natl. Acad. Sci. (USA).* 1988;85:77397742.
- Rosenberg AS, Weatherly B, Rose P and Singer A. Antigen specific effector mechanisms in skin allograft rejection. *Transplant. Proc.* 1989;21:131-132.

Singer A and Rosenberg A. Immune mechanisms of tissue destruction in vivo. In Hamaoka T, (Eds): The Immune System and Cancer. Proceedings of the 19th International Symposium of the Princess Takamatsu Cancer Research Fund. Japan Scientific Societies Press (JSSP), Tokyo, Japan, 1989;pp 211-216.

Gill RG, Rosenberg AS, Lafferty KJ, and Singer A. Characterization of primary T cell subsets mediating rejection of pancreatic islet grafts. J. Immunol. In press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09276-05 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Expression and Function of Porcine Class I MHC Genes in Transgenic Mice

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

PI: D. Singer Senior Investigator EIB, NCI

Others: C. Bordallo Visiting Fellow EIB, NCI  
W. Frels Agricultural Research Service USDA

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.50

## PROFESSIONAL:

2.25

## OTHER:

0.25

## CHECK APPROPRIATE BOX(ES)

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

B

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

Porcine class I major histocompatibility (MHC) genes have been introduced into the genome of C57BL/10 mice in order to study their in vivo patterns of expression and to analyze the ability of their products to function. To date, four transgenic animals containing distinct class I genes have been generated, and demonstrated to transmit the transgene to progeny. One transgenic line which contains a classical class I MHC gene (PD1) expresses SLA antigen on its cell surface and rejects skin of normal C57BL/10 mice, indicating that the foreign SLA antigen is recognized as a functional transplantation antigen. Expression PD1 parallels that observed in the swine, indicating that its expression is regulated. In vivo treatment of the transgenic with  $\alpha, \beta$ -interferon increases PD1 expression in a number of tissues. This increase parallels that observed for the endogenous transplantation antigen, K<sup>b</sup>, but differs markedly from the differentiation antigen, Qa-2.

Two transgenic lines have been generated containing a swine class I gene, PD7, which is closely related to PD1. The two lines differ in the extent of non-coding flanking sequence which has been introduced with the PD7 gene. Although both strains contain the same class I MHC, their patterns of expression of that gene differ. Analysis of PD7 expression in the two lines has revealed the presence of tissue specific enhancers and silencers associated with the gene. The PD7 antigen expressed by both transgenic strains is capable of mediating graft rejection, albeit with differing kinetics.



## Project Description

Major Findings:

Expression of individual class I genes is actively regulated: multiple genes within the family show similar patterns and responses, whereas other class I antigens, such as Qa and TL in the mouse, have been classified as differentiation antigens because of their limited tissue distribution. In order to further define the regulation of these genes, we are constructing transgenic mice, containing individual class I MHC genes from the swine. The swine genome contains seven class I MHC genes. To date, we have generated transgenic animals for three of these genes. All of these have been demonstrated to transmit the transgene and are currently being bred in order to establish lines. Three of these strains, containing the two genes PD1 and PD7, have been sufficiently established to allow studies on the expression of the genes.

Analysis of RNA from swine tissues revealed that both PD1 and PD7 are expressed at highest levels in lymphoid tissues, and at lower but varying levels in a variety of other tissues, including thymus. Within the lymphoid compartment, PD1 expression is differentially regulated, as evidenced by the finding that PD1 is preferentially expressed in B cells. Analysis of RNA from PD1 transgenic mouse tissues reveals that the pattern of PD1 expression, both qualitative and quantitative, parallels that observed in situ. Thus, regulatory elements necessary for the normal expression of PD1 are contained within the 9 kb pig DNA segment which was introduced into the transgenic mouse. The PD1 transcripts present in the transgenic animal are functional, as evidenced by the fact that SLA antigen is observed on splenocytes, lymph node cells, and PBL. Furthermore, skin grafts of transgenic donors onto normal mice are rejected, indicating that the SLA antigen can trigger graft rejection.

Since PD1 is interferon-responsive in transfected mouse L cells, its response to interferon in the transgenic animal was examined. Interferon induced PD1 expression in a variety of tissues, with the largest effects observed in the brain and heart; lower effects were observed in other tissues, with the exception of the kidney where there was no response. Within the lymphoid compartment, PD1 was induced to the same extent in both B and T cells. From these data we conclude that the DNA sequence elements necessary for in vivo interferon response are present within the transgene and that tissue specific elements determine the extent of the response.

Two lines of transgenic mice containing the PD7 gene were generated from two DNA constructs which differed in the extent of 5' flanking sequences adjacent to the patterns of expression were grossly similar between the two. However, there were marked differences in the relative levels of expression of PD7 between the two lines. In some tissues, such as kidney and liver, both constructs were expressed equally. In other tissues, such as heart and skin, the shorter construct was preferentially expressed. The observation that the construct containing additional 5' flanking sequences is expressed less efficiently than a

shorter one suggests the presence of a negative regulatory element associated with the PD7 gene. The fact that equivalent expression occurs in some tissues further indicates that this is a tissue-specific element. In contrast, it was found that in the brain, transgenics containing the longer construct express greater amounts of the PD7 gene, indicating the presence of a brain-specific enhancer.

Both lines of transgenics are capable of mediating graft rejection. Thus, the PD7 antigen is expressed and functions as a transplantation antigen. However, such grafts are rejected more slowly than those containing the PD1 antigen. Furthermore, grafts with the longer PD7 construct are rejected more slowly than those with the shorter construct, consistent with the observed differences in amount of RNA in the skin of the two lines.

Studies are currently underway to identify and characterize the DNA sequence elements which regulate PD7 expression.

Publications:

Ehrlich R, Maguire J and Singer DS. Effects of in vivo interferon treatment on class I MHC gene expression in transgenic mice. *Immunogenetics* 1989; in press.

Singer DS, Frels W and Ehrlich R. Regulation of expression of a class I MHC transgene. in *Transgenic Animals*, ed. N. First, Butterworth Press, Stoneham, MA, 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09277-04 E

## PERIOD COVERED

October 1, 1988 to March 31, 1989

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

Characterization of B Lymphocyte Subpopulations and Membrane Molecules

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

PI: G. Laszlo Visiting Fellow EIB, NCI  
 Others: H. B. Dickler Chief CIB, NIAID

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.8

## OTHER:

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 identify and characterize B lymphocyte subpopulations and membrane molecules. Monoclonal antibody Fl-10 detects a determinant (termed Ly-39.1) preferentially expressed on activated B lymphocytes. Expression peaks 60 hours after activation using LPS. The Ly-39.1 determinant is also expressed on splenic B lymphocytes and a subpopulation of bone marrow cells but at much lower levels and is absent from thymocytes, splenic T lymphocytes, and activated T lymphocytes. Mapping studies using BXD recombinant inbred mice indicate a gene controlling expression of the Ly-39.1 determinant is located on chromosome 17 between hhap-4 and acry-1. These results together with a unique strain distribution pattern suggest that Ly-39.1 is detecting a previously undescribed determinant selectively expressed on activated B lymphocytes which may be a receptor for lymphokines. A new hybridoma screening method has been developed using particle concentration fluorescence which is capable of detecting antibodies binding to molecules which are expressed at very low levels (100-300) molecules per cell). Studies of B lymphocyte subpopulations indicate that large "activated" B lymphocytes obtained directly from mice or B lymphoblasts induced in vitro with F(ab')<sub>2</sub> anti- $\mu$  and lymphokines. Proliferation was augmented 2-4 fold while antibody production was augmented 4-5 fold. This effect was specific for "activated" B lymphocytes in that other cell types did not have this effect. Kinetic experiments revealed that the augmenting signal was effective after stimulation via antigen receptors but prior to the effects of lymphokines. The augmenting effect does not appear to be genetically restricted. Investigation of the nature of the signal revealed that neither supernatants nor plasma membranes from activated B cells alone augmented responses but both together did. These studies suggest that interactions between B lymphocytes are important in regulating humoral immune responses.

## Project Description

Major Findings:

A rat IgG2b, k mAb has been obtained which binds selectively to LPS stimulated B lymphoblasts. A majority of normal spleen B cells and 10-20% of bone marrow cells are also weakly positive (10-20 fold less). Thymocytes, normal T cells from spleen, and Concanavalin A stimulated T lymphoblasts are negative. Expression of the Fl-10 antigen peaks 60 hrs after stimulation with LPS and the kinetics of expression differ from those of sIgM, sIgD, Fc receptors and Ia antigens.

Monoclonal anti-Fc $\gamma$ R and IgG anti-MHC class II antibodies inhibit binding of Fl-10, while IgG anti-class I and IgM anti-class II antibodies do not. However, Fc $\gamma$ R and Fl-10 expression are discordant on certain tumor cell lines as well as in the kinetics of expression after LPS stimulation. Thus, these 2 molecules are probably not identical but may be associated.

Typing of LPS blasts from various mouse strains for Fl-10 expression reveal that DBA/2, DBA/1, AKR/J, NZB, MRL, and BXSB are intermediate or high expressors while C55BL/10, A/J, Balb/c, and C57L are low or negative for expression. This is a unique strain distribution pattern.

Mapping studies using BxD recombinant inbred mice reveals that a gene controlling expressing of the Fl-10 determinant is located on chromosome 17 between hbaps-4 (3 mismatches in 22 strains) and acry-1 (3 mismatches in 20 strains).

It was concluded from the tissue distribution, kinetics of expression, strain distribution, and mapping studies that Fl-10 detects a previously undescribed B lymphocyte membrane molecule. The data are consistent with the possibility that this molecule is a lymphokine receptor.

Many molecules of importance on the B lymphocyte surface may be expressed at very low levels. Thus, it is important to develop a method which detects monoclonal antibodies specific for such molecules. We have recently successfully adapted particle concentration fluorescence immunoassay for use as a screening procedure. For this assay, lymphocyte aliquots are first incubated with supernatants from the clones to be screened (or known rat antibodies as controls) followed by FITC-mouse anti-rat Ig. The labeled cell samples are then loaded into specially designed plates which have 96 wells each of which has a 0.2 filter bottom. Protein coated 3.4 polystyrene beads are added to allow efficient filtration, the cells are filtered onto the bottom of the wells by application of vacuum, and total cell bound fluorescence in each well rapidly determined by front-surface fluorometry (i.e. fluorometry of the surface of the filter). Using rat monoclonal antibodies specific for known B lymphocyte surface molecules, we have been able to detect as few as  $10^7$  (and reproducibly  $3 \times 10^7$ ) molecules among  $10^5$  cells so that theoretically we can identify monoclonal antibodies which are specific for molecules whose expression is as low as 100-300 molecules per cell.



This screening system has been used to identify several rat monoclonal antibodies which appear to bind to activated but not resting B lymphocytes. These antibodies are currently being further characterized.

Purified (96% sIg positive, 0.1% T cells, 0.1% macrophages) small (120<sup>3</sup> diam) dense (>1.087) normal B lymphocytes are poor responders to a variety of polyclonal stimulators including LPS and F(ab')<sub>2</sub> 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<sup>3</sup> diam) less dense (1.075-1.081) normal B cells have higher background proliferation and antibody production, are brisk responders to the above stimuli and are considered "activated". Addition of the "activated" B cells (irradiated with 4000 R to prevent responses) to cultures of "resting" B cells stimulated with F(ab')<sub>2</sub> 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')<sub>2</sub> 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 hrs after but not 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<sub>1</sub>, or mixtures of B6 and DBA/2. Finally, anti-Ia antibody did not block augmentation.

Supernatants from activated B cells were prepared as well as plasma membranes from these cells and both were tested for augmenting activity. Neither the supernatant nor the membranes alone provided augmentation. However, a mixture of both did reconstitute the augmenting activity, while activated B cell supernatant plus thymocytes or thymocyte plasma membranes did not. Thus, the augmenting activity appears to have two components, one soluble and one membrane bound.

#### Publications:

Park E, Principato MA, Lamers MC, Dickler HB. Ly-39: a new lymphocyte alloantigen defined with a monoclonal antibody. Immunogenetics 1988;28:64-66.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09279-04 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Isolation and Characterization of a Novel H-2 Class I Gene

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

PI: Dinah Singer Senior Investigator EIB, NCI

Others: S. Rudikoff Senior Investigator LG, NCI

K. Howcroft IRTA EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

A previously unidentified sub-family of H-2 class I genes, consisting of at least two members, has been identified in the genome of the C57BL/10 mouse. One of these novel genes, Mb1, has been extensively characterized. Using a series of recombinant strains of mice, Mb1 has been mapped telomeric to the Qa locus. DNA sequence analysis of Mb2 demonstrates that the exonorganization of this gene resembles that of other class I genes. However, the level of DNA sequence homology of Mb1 to other H-2 genes is no greater than to human, pig, or rabbit class I genes. Furthermore, the over-all organization of Mb1 is more similar to man and pig than to mouse. These data suggest that Mb1 may represent a direct descendant of a primordial class I gene, which antedates speciation.

Despite the extensive divergence of Mb1 from the rest of the H-2 class I gene family, Mb1 has open reading frames and legitimate splice sites throughout, enabling it to encode a transmembrane protein. However, Mb1 is not detectably expressed in a variety of cell lines or adult somatic tissues. The only evidence for possible Mb1 expression is in activated T cells. Introduction of full-length Mb1 into mouse L cells does not result in its transcription. However, constructs containing truncated 5' flanking sequences are expressed.

## Project Description

Major Findings:

Many of the class I MHC gene of the mouse have been previously identified and characterized as transplantation antigen genes or closely related differentiation antigen genes. However, there is at least one sub-family of class I genes which is highly divergent from the rest. We have conducted a careful study of the structure and expression of one of the members, Mbl, of this new sub-family.

DNA sequence analysis of Mbl revealed that it consists of five exons, encoding a leader, three extracytoplasmic domains, and a transmembrane domain and intracytoplasmic anchor. No exons 6,7, or 8 were found. All exons contain open reading frames and conserved splice sites. The gene terminates in a poly A addition site. At the 5' end, canonical promoter elements were found. Comparison of the Mbl sequence with other class I genes revealed an average homology of about 60% in the exons and about 30% in the introns. Mbl is no more related to mouse class I genes than to those of other species. Analysis of the exon/intron organization of Mbl gave rise to the surprising finding that Mbl is more like non-murine genes than other murine genes. These observations lead to the speculation that Mbl has evolved from an ancestral gene which antedates speciation and that Mbl has not participated in the various gene conversion events that have occurred within the rest of the class I family.

Using a series of recombinant mice, it has been possible to demonstrate that Mbl maps to the right of the Qa locus. The precise location is unknown, since the appropriate recombinants are not available.

Although Mbl by DNA sequence analysis appears to have the capacity encode a product, it has not been possible to identify such a product. Mbl transcripts are not detected by direct Northern analysis in a variety of lymphoid and non-lymphoid lines which have been analyzed. Adult somatic tissue similarly does not express Mbl. The only cell type in which some Mbl expression is detected is in activated T cell populations. The relationship between Mbl expression and T cell activation is currently under investigation. Introduction into L cells of the Mbl gene within the 17 kb genomic fragment of DNA in which it was isolated does not result in the expression of the gene. However, introduction into L cells of a 3.5 Kb derivative, containing all of the Mbl coding sequences, does generate Mbl transcripts. These observations suggest the present of a negative regulatory element associated with Mbl flanking sequences. The product of Mbl remains to be identified; the function of this product, if any, remains to be determined.

Publications:

Singer D, Hare J, Golding H, Flaherty L, and S. Rudikoff. Characterization of a new subfamily of class I genes in the H-2 Complex of the mouse. Immunogenetics 1988;28:3-21.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09281-04 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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	Senior Investigator	EIB, NCI
Others:	K. S. Hathcock	Chemist	EIB, NCI
	D. M. Segal	Senior Investigator	EIB, NCI
	M. Taplits	Medical Staff Fellow	EIB, NCI
	P. Bohjanan	Howard Hughes Fellow	EIB, NCI
	K. Kelly	Senior Investigator	EIB, NCI

## COOPERATING UNITS (if any)

Food and Drug Administration

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

Immunotherapy Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

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

The 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 V region 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 V $\beta$ 8 determinants on the T cell receptor. Naive Lyt2<sup>+</sup> T cells were activated to proliferate in the presence of soluble F23.1, IL-2, and accessory cells. Under the same conditions, L3T4<sup>+</sup> 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<sup>+</sup> and L3T4<sup>+</sup> T cell subpopulations.

The role of endogenous lymphokines in T cell activation has been demonstrated. The proliferative response of type 2 T helper clones is inhibited by antibody specific for the lymphokine IL4, which is produced by these clones. This demonstrates that IL4 acts as an autocrine growth factor for these T cells. In addition, IL4 mRNA levels corresponding to a number of activation genes are inhibited by the presence of anti-IL4 antibody during T cell activation. In contrast, the level of mRNA is actually increased in the presence of this antibody, suggesting that an endogenously produced IL4 may normally down regulate its own gene transcription. The regulation of IL3, IL4, IL5, and GM-CSF lymphokine gene expression by type 2 helper clones was studied in more detail, using multiple inducing stimuli and pharmacologic inhibitors. It was found that independent (non-coordinate) regulation of discrete lymphokine genes can occur and is dependent upon the nature of the inducing stimulus.



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 Thyl.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<sup>+</sup> 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<sup>+</sup> T cells. In contrast, the Fab of F23.1 covalently linked to the Fab on anti-A<sup>k</sup><sub>α</sub>A<sup>k</sup><sub>β</sub> antibody was capable of activating Lyt2<sup>+</sup> or L3T4<sup>+</sup> T cells even in the absence of IL-2 but in the presence of A<sup>k</sup><sub>α</sub>A<sup>k</sup><sub>β</sub>-positive accessory cells. This activation mediated by heteroaggregate antibody required specific targeting by covalently linked antibodies, and was not due to the independent effects of F23.1 and anti Ia antibodies. Proliferation of naive T cells was also induced by antibodies to a component of the T3 complex, in the absence of exogenous lymphokine. Under these conditions, proliferation was substantially inhibited by antibodies to IL-2 or to IL-4, suggesting a possible role of endogenously produced lymphokines in the proliferative response of T cells to this stimulus. Activation of naive T cells was also assessed by the measurement of gene induction. Increased levels of myc and IL-2 receptor mRNA were induced in response to anti-T3, F23 + IL-2, or Con A.

## 2. Activation of Cloned T Cells.

F23.1 (Vβ8) expressing T cell clones could be triggered to proliferation in the presence of F23.1 soluble antibody and accessory cells. In these circumstances, there was no requirement for specific recognition of MHC determinants on accessory cells by responding T cell clones. Re-targeting by heteroconjugate antibodies also produced enhanced proliferative responses by T cell clones and was again specific for cross linking of T cell receptor structures to determinants on the surface of accessory cells. F23.1 antibody immobilized either on plastic tissue culture wells or on polystyrene beads was capable of activating cloned T cells in the absence of accessory cells or exogenous lymphokines.

## 3. Function of Endogenous Lymphokines in T Cell Activation.

Cloned T helper cells produce a number of now well characterized lymphokines and in addition are themselves responsive to a number of lymphokines. One class of T cell clones, termed type 2, produces IL4 and is induced to proliferate in response to exogenous IL4. The fact that IL4 acts as an autocrine growth factor for these cells was demonstrated. Proliferation and T cell activation were induced by immobilized antibodies specific for the T3 complex of the T cell receptor. Under these conditions, proliferation was blocked by the presence of antibody to IL4. Activation of other genes including the proto-oncogenes c-myc and c-myc were also inhibited by the presence of anti-IL4 antibody. T cell activation mediated through the T cell receptor complex is thus dependent upon an autocrine role of IL4. In contrast to these inhibitory influences, it was

observed that the presence of anti-IL4 antibody resulted in a significant increase in steady state IL4 mRNA levels. These latter data suggest that the presence of endogenously produced IL4 normally down regulates the production of further IL4, and that it does so at the level of gene transcription or of message stabilization. It was found that the pattern of lymphokine genes expressed by type 2 T helper clones was dependent upon the stimulus used to activate the T cells. Some lymphokines, such as IL5, were induced in response to anti-CD3 as well as in response to recombinant IL2 stimulation, whereas IL4 gene expression by the same clone was induced by anti-CD3 but not recombinant IL2. Studies employing the pharmacologic agents cyclohexamide and cyclosporine A confirmed that IL4 and IL5 genes could be induced through independent 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. The role of exogenous as well as endogenously produced lymphokines on T cell proliferation and specific gene induction will also be evaluated.

#### Publications:

Hathcock KS, Segal DM, and Hodes RJ. Activation of Lyt2<sup>+</sup> (CD8<sup>+</sup>) and L3T4<sup>+</sup> (CD4<sup>+</sup>) T cell subsets by anti-receptor antibody. J. Immunol. 1989;142:2181-2186.

Bonvini E, DeBell KE, Kolber ME, Hoffman T, Hodes RJ, and Taplits MS. Hydrolysis of inositol phospholipids induced by stimulation of the T cell antigen receptor complex in antigen-specific, murine T cell clones: requirement for exogenous calcium. J. Immunol. 1989, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09282-03 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

T Cell Immune Deficiency in Mice and Humans With Autoimmune Disease

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

PI: G. M. Shearer Senior Investigator EIB, NCI

Others: G. C. Tsokos Senior Investigator ARB, NIADKDK

## COOPERATING UNITS (if any)

C. S. Via Dept. of Medicine, University of Maryland, School of Medicine,  
Baltimore, MD

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.4

## PROFESSIONAL:

0.2

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

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

B

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

Spleen cells from Mrl-lpr mice gradually develop a loss of CD4<sup>+</sup> T helper cell function that is age-dependent which is concomitant with the appearance of anti-DNA antibodies and of suppressor cells that selectively suppress only CD4<sup>+</sup> T helper cell function. These suppressor cells were found to be T lymphocytes of the L3T4 phenotype. This observation contrasts with MRL +/+ mice that exhibit none of the above effects were observed during the same time period.

Sixteen of twenty human patients with SLE exhibit a similar selective loss of CD4 T helper cell function, which suggests a similarity between the immunological changes that occur in the murine lpr model of autoimmunity and SLE in humans.

## Project Description

Major Findings:

Based on our findings that mice undergoing the autoimmune form of GVH (see #05088) exhibit a selective defect in CD4<sup>+</sup> T helper cell function, and generate suppressor cells that are selective for CD4<sup>+</sup> T helper cells, we have tested whether mouse strains that make anti-DNA antibodies naturally exhibit a selective defect in CD4<sup>+</sup> T helper cell function and generate suppressor cells that selectively suppress CD4<sup>+</sup> T helper cells. We found that spleens of two-month old MRL-lpr mice exhibited normal T cell functions, whereas four- and six-month old MRL-lpr mice exhibited both deficient CD4<sup>+</sup> T helper cell function and suppressor cell activity selective for CD4<sup>+</sup> T helper cells. MRL +/+ mice of the same ages did not exhibit these defects. These results raise the possibility that anti-DNA antibody production activates this unusual suppressor cell population which is an attempt to down-regulate CD4<sup>+</sup> T helper cell function.

Recent studies suggest that other models of B cell hyperactivity including B cell activation by lipopolysaccharide or the natural B cell hyperactivity that occurs in aging SJL/J mice are associated with selective suppression of CD4<sup>+</sup> T helper cell function. Studies with PBL from patients with SLE also exhibited a loss in CD4<sup>+</sup> T cell function in 16 to 20 patients tested.

Publications:

Via CS, Shearer GM. T-cell interactions in autoimmunity: Insights from a murine model of graft-versus-host disease, *Immunol. Today* 1988;9:207-213.

Via CS, Shearer GM. T cell immunity to hapten-modified self in normal and autoimmune conditions. In R.W. Estabrook, E. Lindenlaub, F. Oesch, and A. DeWeck (Eds.): *Toxicological and Immunological Aspects of Drug Metabolism and Environmental Chemicals*. Symp. Medica Hoechst 22, F.K. Schattauer Verlag:Stuttgart, 1988; pp 185-194.

Via CS, Shearer GM. Autoimmunity and the acquired immunodeficiency syndroms. *Current Opinion of Immunology* 1989; in press.

Via CS, Shearer GM. Functional heterogeneity of L3T4<sup>+</sup> T cells in MRL-lpr/lpr mice. L3T4<sup>+</sup> T cells suppress major histocompatibility complex self-restricted L3T4<sup>+</sup> T helper cell function. *J. Exp. Med.* 1988;168:2165-2181.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09283-03 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Regulation of Human T Cell Responses by Adherent Cells

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

PI: G. M. Shearer Senior Investigator EIB, NCI

Others: S. Muluk Medical Staff Fellow EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

B

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

Depletion of human peripheral blood leukocytes (PBL) of Leu M3<sup>+</sup> cells by adherence to plastic and Sephadex G10 results in the abrogation of proliferative (<sup>3</sup>H) and cytotoxic T lymphocyte (CTL) responses to influenza A virus (FLU), but in an elevation of <sup>3</sup>H and CTL responses to HLA alloantigens (ALLO). This loss of the FLU T cell response was attributed to removal of antigen-presenting cells (APC), whereas the elevation of the ALLO response was shown to be due to a suppressor cell (or a suppressor inducer cell) that is contained in the Leu M3<sup>+</sup> adherent cell population. Suppressor activity was inactivated by culturing either unfractionated PBL or adherent cells with viruses, including influenza A, adenovirus or vaccinia virus. The suppressor activity was attributed to a soluble factor produced by Leu M3<sup>+</sup> cells.

## Project Description

Major Findings:

PBL from HIV<sup>+</sup> donors were studied for their ability to generate proliferative and CTL responses to HLA alloantigens. PBL from approximately 70% of HIV<sup>-</sup> donors generated weak or only marginal CTL responses to ALLO, whereas they generated strong CTL responses to FLU in the same experiments. PBL from the other 30% of donors generated strong ALLO CTL responses. The phenomenon was donor-selective in that those donors who were poor CTL responders to ALLO remained poor responders for more than two years, and poor responsiveness was observed irrespective of the source of ALLO stimulators and irrespective of whether a single donor or a pool of ALLO stimulators was used. Proliferative responses to ALLO were also reduced, but not as much as CTL responses. The ALLO CTL responses of these weak responders was elevated to the levels observed for FLU-specific CTL in the same donors and to the levels observed for ALLO responses in many HIV<sup>+</sup> donors and AIDS patients, by procedures that removed macrophages from the responder population. Addition of irradiated, autologous unfractionated PBL or irradiated, autologous macrophage-enriched PBL suppressed the level of CTL activity to that of unfractionated PBL.

The fact that this suppression appeared to affect ALLO CTL, but not FLU CTL activity prompted us to question whether influenza virus in some way abrogated suppressor cell activity. We found that suppressor cell activity was abolished by incubation of PBL with influenza virus, as well as by adenovirus or vaccinia virus. Furthermore, we found that the suppression was mediated by a filterable, soluble material that is produced by the macrophages.

Publications:

Bernstein DC, Shearer GM. Suppression of human cytotoxic T lymphocyte responses by adherent peripheral blood leukocytes. Ann. N.Y. Acad. Sci. 1988;532:207-213.

Muluk SC, Bernstein DC, Shearer GM. A virus-sensitive suppressor cell is involved in the regulation of human allospecific T cell immunity. J. Immunol. 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09285-03 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Induction of Class I MHC Gene Expression by Ethanol

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

PI: Dinah Singer Senior Investigator EIB, NCI

Others: M. Wallendal Guest Researcher EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.75

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

Ethanol enhances expression of cell surface class I major histocompatibility complex (MHC) antigens in a variety of cell lines; up to an eightfold increase is observed in an embryonic cell line. Increased cell surface expression, following alcohol treatment, occurs with a concomitant increase in steady-state RNA levels. Measurement of class I MHC antigen on peripheral blood lymphocytes in acutely ethanol intoxicated patients showed a highly significant increase over controls. Ethanol treatment of mice results in elevated blood alcohol levels and markedly changes in both class I MHC expression and splenic cellularity. The alcohol response in mice consists of two temporally distinct phases, one of which is genetically controlled.

## Project Description

Major Findings:

Treatment of a variety of cell lines, including thymomas, fibroblasts, and embryonic cells, with ethanol increases the level of class I MHC gene expression on their cell surface. It has been demonstrated that intracellular protein synthesis increases, as do steady state RNA levels.

These observations led to the speculation that in vivo, ethanol may also induce increased levels of class I MHC gene expression, which might lead to auto-immune components in alcohol-related diseases. A group of acutely intoxicated patients was tested for PBL class I MHC levels. It was found that there is a significant increase in the level of cell surface expression of class I antigens in the patient population, relative to normal controls. In contrast to the observations in cell culture, no increases in class II levels were noted.

To pursue the in vivo effects of ethanol, a murine model of alcoholism has been established. Congenic mouse strains, which differ only in their MHC locus, were found to differ in their responses to ethanol ingestion. Whereas both strains of mice consume equivalent volumes of Lieber-DiCarli diet and have indistinguishable blood alcohol levels (250-350 mg/dl) during the first two weeks of treatment, splenocytes from the B10.A strain show marked decreases in class I expression whereas there is no change in class I levels of B10 splenocytes. After three to four weeks, an elevation in splenic class I levels is observed in both strains of mice. However, the treated B10.A mice never exceeded the levels of the matched controls, whereas the B10 mice did.

By other parameters, the two strains of mice are indistinguishable in their responses. In both the B10 and B10.A mice, a 10-fold depletion in spleen cell number occurs within the first 2-3 weeks of ethanol-treatment. This depletion largely spares Thyl<sup>+</sup> cells, namely NK cells and T lymphocytes, which increase from 15-20% of the total spleen population to 50%. The spleen cell loss is accompanied by a dramatic shrinkage in size. By 3-4 weeks, the spleen has been repopulated such that it contains as many cells, if not more, than the control and has increased to its original size. Increased class I mRNA levels occur in both strains of mice in liver, but not in brain.

Publications:

Kolber M, Walls R, Hinners M, and Singer D. Evidence of increased class I MHC expression on human peripheral blood lymphocytes during acute ethanol intoxication. *Alcoholism: Clinical and Experimental Research* 1988;12:820-822.

Singer DS, Parent LJ, Kolber MA. Ethanol: An enhancer of transplan- tation antigen expression. *Alcoholism: Clinical and Experimental Research* 1989; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09287-02 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Marrow Graft Failure in Allogeneic Bone Marrow Transplantation

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

PI:	R. E. Gress	Senior Investigator	EIB, NCI
Others:	H. Nakamura	Visiting Fellow	EIB, NCI
	R. Hirsch	Clinical Associate	EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B, D

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

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL) may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system, CTL were cloned from the spleens of sublethally irradiated animals which had rejected MHC disparate marrow grafts. It was found that cloned CTL were sufficient to effect rejection of T cell depleted allogeneic marrow in lethally irradiated animals. The rejection of marrow grafts by CTL was specific for the MHC gene products expressed by the marrow cells and correlated with the cytotoxic specificity of the individual clones. Since CTL can therefore reject marrow grafts, the administration of anti-CD3 monoclonal antibody in vivo has been studied as a means to enhance engraftment of T cell depleted marrow in sublethally irradiated murine hosts. Cloned xenospecific human CTL have also been directly evaluated for their ability to mediate tissue damage in vivo and were found to mediate tissue injury with specificity for MHC antigens of the murine host. The fine specificity of xenoantigen recognition by human T cells suggest that in transplantation of xenogeneic tissue, as in transplantation of MHC mismatched allogeneic tissue, the particular tissue antigens, and therefore tissue typing, may be a relevant consideration. The generation of human CTL has been characterized and certain essential cellular interactions in that generation have been identified. In addition, the mechanisms for the blocking effects of monoclonal antibodies specific for the CD2 (sheep red blood cell receptor), CD8, and CD18 (LFA-1) molecules expressed on the surface of human CTL have been investigated. Results indicate that the inhibition by anti-CD2 antibody did not occur in the absence of target cells, consistent with the interpretation that this antibody exerts its blocking effect by interfering with a CD2-ligand interaction. However, results with anti-CD8 and anti-CD18 monoclonal antibodies indicated that inhibition by these agents could not be explained by simple disruption of a T cell receptor-target cell ligand interaction.

## Project Description

Major Findings:

The purpose of these studies was to directly assess the ability of murine CTL from irradiated hosts to reject allogeneic marrow grafts. First, the maximum dose of radiation which still allowed an effective rejection response by the host was determined. By an  $^{125}\text{I}$ UDR uptake assay of splenic marrow cell proliferation, this dose was found to be 650-750 cGy. Lymphocytes were then isolated and cloned from spleens of B6(H-2<sup>b</sup>) mice seven days after they had received 650 cGy radiation and inoculation with either BDF1 (H-2<sup>b/d</sup>) or B6C3F1 (H-2<sup>b/k</sup>) marrow which had been depleted of T cells by rabbit anti-mouse brain antiserum plus complement. The clones which were recovered were Thy 1+, LyT 2+, L3T4- with cytolytic specificity for the allogeneic MHC-encoded determinants of the marrow donor. No NK activity could be identified. It was found that these clones suppressed MHC mismatched marrow graft proliferation (measured by  $^{125}\text{I}$ UDR uptake) when adoptively transferred into a 1025 cGy lethally irradiated B6 host if and only if the grafted marrow cells expressed MHC determinants for which the individual clone had cytotoxic specificity. Two primary questions were then addressed. The first was the effect of these CTL cells on long term engraftment and the second was the mechanism by which they exerted their effect. Results demonstrated that (1) a cloned CTL population is sufficient to reject an allogeneic marrow graft, and (2) the mechanism by which these marrow grafts were rejected was specific for MHC gene products expressed by the donor marrow corresponding to the cytotoxic specificity of the clone.

With the demonstration that CTL are sufficient to effect marrow graft rejection, a study was undertaken to evaluate the possible beneficial effects of anti-CD3 treatment on marrow engraftment in a mouse model (parallel to anti-CD3 monoclonal antibody treatment which is currently available for clinical use in allogeneic organ transplantation). The antibody used is specific for the  $\epsilon$  chain of the murine CD3-T cell receptor complex, can suppress skin graft rejection, and can cause both short term and long term in vivo T cell dysfunction. T cell immunosuppression is pronounced at one week after administration of the antibody. It was found, however, that in vivo treatment with anti-CD3 administered seven days before infusion of bone marrow did not enhance engraftment of allogeneic marrow in sublethally irradiated hosts. Therefore, immunosuppression provided by treatment with anti-CD3 monoclonal antibody was not sufficient to prevent rejection of the marrow graft. More recent studies have demonstrated that administration of anti-CD3 to mice results in T cell activation within hours of administration, manifest by increased IL-2 receptor expression and by enhanced proliferation of spleen cells from treated animals to exogenous IL-2 in vitro. This activation also results in secretion of colony stimulating factors (CSF) detectable in the serum and produces extramedullary hematopoiesis in the spleen. These results indicate that it may be beneficial in terms of marrow stimulation and engraftment to time the injection of allogeneic marrow so that it is present during this period of T cell activation in order to provide the possible benefit of factors which stimulate hematopoiesis.

Information about human CTL generation, specificity, and in vivo function is not as extensive as in the case of murine CTL. These studies of human anti-mouse CTL responses indicate that both CD4+ and CD8+ helper pathways function in the generation of human CTL responses and that there exists a dependence on the presence of human antigen presenting cells. Additional experiments were carried out to characterize the human effector CTL subsequent to its generation. CTL lines and clones were established from a secondary mixed lymphocyte response in which the responder cells were human and the stimulators were H-2<sup>b</sup> murine splenocytes. These human xenoreactive CTL were able to distinguish wild type K<sup>b</sup> molecules from those altered in their amino acid sequence by in vitro mutagenesis. Such amino acid changes were confined to a single domain and so demonstrated a fine specificity of recognition by human CTL comparable to the specificity of recognition of K<sup>b</sup> determinants by alloreactive murine CTL. This fine specificity of antigen recognition by human CTL across species predicts that the recognition by host CTL of MHC encoded antigens expressed by a xenograft would be expected to be comparable in specificity to the recognition of MHC antigens expressed by an MHC mismatched allograft.

These xenoreactive CTL populations were also used to evaluate whether isolated human CTL can mediate tissue injury in vivo. Human CTL were injected intradermally into murine hosts which either did or did not express the MHC determinants for which the cytotoxic populations were specific. In all instances in which an anti-H-2<sup>b</sup> human CTL line or clone was injected into an H-2<sup>b</sup> host, anatomical and histological changes developed. Anatomical lesions were first detected at 48 hours with progression to eschar formation by 96 hours. Histologically, exocytosis and toxic epidermal necrolysis was present at 48 hours with lymphocytic infiltration into the epidermis.

To investigate the blocking of CTL function by monoclonal antibodies, we have carried out blocking studies with CD2, CD3, CD8, and CD18 specific monoclonal antibodies in vitro. Each of these antibodies demonstrated some ability to inhibit the generation or function of CTL. The CD2, CD8, and CD18 molecules expressed on T cells are generally felt to function through the binding of ligands expressed by target cells. These ligands are LFA-3, Class I MHC determinants, and ICAM-1, respectively. The ability of monoclonal antibodies specific for CD2, CD8, and CD18 to block CTL function is therefore felt to be through a disruption of the normal interactions of these molecules with ligands on the stimulator/target cell. To investigate whether disruption of receptor-ligand interaction is in fact the mechanism of action of blocking by these agents, an assay of CTL function was developed which is independent of target cells. Two antibodies blocked in this system, anti-CD8 and anti-CD18. The ability of anti-CD8 to block, together with results obtained in blocking the killing of Class I negative targets, makes it unlikely that the mechanism of inhibition depends on a disruption of a CD8-target cell Class I interaction. In addition, a CD8+, Class II specific CTL clone, which was also blocked in the target cell-independent assay system, was not blocked by anti-CD8 monoclonal antibody in its lysis of target cells expressing the Class II antigen for which it is specific, suggesting that CD8 specific antibody does not inhibit CTL function by generating an absolute off signal. There was profound inhibition by anti-LFA-1 (CD18). Secretion induced by ionomycin +/- PMA was not inhibited, thereby demonstrating that the antibody did not act at the level of secretion.

These experiments strongly suggest that anti-LFA-1 inhibition, which has been utilized to a limited extent clinically in allogeneic bone marrow transplantation, may involve more than a disruption of the CD18 - ICAM-1 pathway of adhesion.

Publications:

Hirsch R, Gress R, Pluznik DH, Eckhaus M, and Bluestone JA. Effects of in vivo administration of anti-CD3 monoclonal antibody on T cell function in mice II. In vivo activation of T cells. *J. Immunol.* 1989;142:737-743.

Quinones RR, Segal DM, Henkart P, Perez P, and Gress RE. Anti-CD8 monoclonal antibody inhibition of cytotoxic T cell lysis of nominal antigen negative targets: studies with CD-3 redirected cytotoxicity. *J. Immunol.* 1989;142:2200-2206.

Hirsch R, Chatenoud L, Gress RE, Bach JF, Sachs DH, and Bluestone JA. Suppression of the humoral response to anti-CD3 mAb by pretreatment with anti-CD4. *Trans. Proc.* 1989;21:1015-1016.

Hirsch, R., Chatenoud, L., Gress, R.E., Sachs, D.H., Bach, J.F., and Bluestone, J. Suppression of the humoral response to anti-CD3 monoclonal antibody. Transplantation. In press.

Gress, R. E., Nathenson, S. G., and Lucas, P. L. Fine specificity of xenogeneic antigen recognition by human T cells. Transplantation. In press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09288-02 E

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

T Cell Responses in Bone Marrow Transplantation

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

PI: R. E. Gress Senior Investigator EIB, NCI

Others: R. Moses Biotechnology Fellow EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B,D

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

Concerns that a functionally effective T cell repertoire might never develop in the setting of MHC-mismatched marrow transplantation are based on the defective T cell responses in vivo observed in murine bone marrow chimeras constructed between MHC disparate strains. The incompetence is not due to inherent T cell defects, but rather to the restriction specificities of the T cells such that those MHC determinants recognized as self (host type) are not expressed by the antigen presenting cells which are of donor type. The T cell response to antigen is therefore defective. To evaluate whether T cell maturation is comparable in mouse and man, the development of T cell responses in a primate, the rhesus monkey, has been studied. The biology of T cell recovery has first been investigated in animals following T cell depleted autologous marrow transplantation. The time course of CD4+ T cell reconstitution and the time course of the development of in vivo T cell immunocompetence as defined by the ability to respond to an organ allograft correlated with the number of T cells infused in the marrow, and did not correlate with marrow cell dose or with time of hematopoietic reconstitution, raising the possibility that residual T cells in the infused T cell depleted marrow played a central role in the generation of subsequent T cell populations. This would imply that the generation of T cells in marrow grafted primates may not be the same as in the mouse.

## Project Description

Major Findings:

As a preliminary step to the study of T cell repertoire generation in MHC-mismatched BMT, we have studied the generation of T cell function in rhesus monkeys which have been hematologically reconstituted with either untreated or extensively T cell depleted autologous bone marrow. The methods for T cell depletion and the quantitation of residual (reinfused) T cells were patterned after methods developed for carrying out bone marrow transplantation in man. By phenotypic analysis, CD2+/CD8+ T cells recovered by 6-8 weeks post grafting. CD16+ NK cells and CD20+ B cells also recovered at 6-8 weeks. All animals receiving T cell depleted marrow recovered CD4+ cells at later time points. In the animal receiving marrow containing the fewest residual T cells (0.00014% by limiting dilution assay), CD4+ cells were less than 30% of the pretransplant value at ten months after transplant. The total number of T cells required to acutely reject a graft was determined on the basis of results in animals receiving marrow poorly depleted of T cells, and was  $5 \times 10^4$  per kilogram recipient body weight. In vitro functional studies paralleled the phenotypic analysis. CTL activity, which can be mediated by CD8+ cells, was observed in vitro (with added IL-2) at six weeks post transplant; recovery of helper T cell function, predominately mediated by CD4+ cells, was found only at time points beyond four months. The ability to respond to allogeneic stimulation in vivo as assessed by the rejection of an organ allograft temporally paralleled the recovery of CD4+ helper cells, not the recovery of CD8+ cytotoxic cells. The critical level of recovery of peripheral CD4+ cells which correlated with allograft rejection was 25-50% of the pretransplant value. Approaches to evaluate T cell function by direct stimulation of CD3 were also developed and characterized.

The pattern and time course of reconstitution of T cell populations establishes a baseline for comparing T cell reconstitution in allogeneic donor/recipient combinations and in predicting the time required for successful treatment to reverse virally induced T cell deficiencies. Prolonged periods of depressed T cell number and function existed even in the absence of the allogeneic effects which might be expected in allogeneic BMT. The slow rate of recovery of CD4+ cells as compared to CD8+ cells may reflect distinct mechanisms in the generation of these two subsets. Also, even with very extensive T cell depletion, hematopoietic recovery occurred, indicating that hematopoiesis does not absolutely require many, if any, "hematopoietic T helper cells".

The length of time required for reconstitution of CD4+ cells and for recovery of organ allograft rejection varied inversely with the number of residual T cells in the infused marrow, not with stem cell function as assessed by the number of marrow cells infused and by rapidity of overall hematopoietic recovery. This result is consistent with the possibility that the residual T cells in the infused marrow play a central role in the generation of subsequent T cell populations in the recipient. This suggests that the generation of T cells in the primate marrow recipient may in fact be different than in the mouse where

the T cell repertoire appears to develop from marrow precursor cells which mature in the irradiated recipient and which consequently express an MHC restriction which is strongly influenced by the host environment. The possibility that reconstituting T cells in the primate following marrow transplantation are derived from mature donor T cells (with restriction specificity for donor MHC antigens) remaining in the marrow after depletion rather than from early precursors/stem cells is of central importance to considerations of MHC mismatched BMT in man. These findings are being pursued by molecular analysis of the reconstituting T cell populations.

Publications:

Moses RD, Orr KS, McVittie TJ, and Gress RE. Experimental cardiac allograft survival across MHC barriers in the rhesus monkey following autologous bone marrow transplantation. I. In vitro T lymphocyte depletion studies. Transplantation 1988;46:197-205.

Sundt TM III, Suzuki T, Kortz EO, Eckhaus M, Gress RE, and Sachs DH. Induction of specific tolerance in a large animal model by bone marrow transplantation. Surgical Forum 1988;39:365-367.

Moses RD, Orr KS, Bacher JD, Sachs DH, Clark RE, and Gress RE. Cardiac allograft survival across MHC barriers in the rhesus monkey following T lymphocyte-depleted autologous marrow transplantation II. Prolonged allograft survival with extensive marrow T cell depletion. Transplantation 1989;47:435-444.

Golding H, Shearer GM, Hillman K, Lucas P, Manischewitz J, Zajec RA, Clerici MM, Gress RE, Boswell R, and Golding B. Common epitope in HIV I-GP41 and HLA class II elicits immunosuppressive autoantibodies capable of contributing to immune dysfunction in HIV I infected individuals. J. Clin. Invest. 1989;83:1430-1435.

Suzuki T, Sundt TM, Kortz EO, Mixon A, Eckhaus M, Gress RE, Spitzer T, and Sachs DH. Bone marrow transplantation across an MHC barrier in miniature swine. Trans. Proc. 1989;21:3076-3078.

## SUMMARY

### ANNUAL REPORT OF THE LABORATORY OF CELL BIOLOGY OCTOBER 1, 1988 TO SEPTEMBER 30, 1989

The following are selected highlights:

The 3-methylcholanthrene-induced murine fibrosarcoma, Meth A, has been shown to display at least three different tumor-specific transplantation antigens (TSTA). One of the TSTA is an 82-kDa cytosolic protein that is also present in nontransformed cells. Another of the TSTA has been putatively identified by another laboratory as the 96-kDa glucose-regulated protein (but see later information). The third TSTA is a mixture of two heat shock proteins, designated HSP84 and HSP86. These latter proteins have been studied extensively in this laboratory. HSP84 and HSP86 are coded for by separate, but related, genes. Full-length cDNA clones have been isolated and their nucleotide sequences determined. The deduced protein sequences of HSP84 and HSP86 are 724 and 733 amino acid residues, respectively. The two proteins are 86% homologous, whereas their cDNA coding regions are 76% homologous. The genes coding for these proteins apparently arose by a gene duplication some 500 million years ago. HSP-related sequences comprise a family of genes, three of which were assigned to chromosomes 17, 12, and 2. The HSP structural gene has been mapped to 5.6 cM telomeric to the major histocompatibility complex (MHC) class I region on chromosome 17. The HSP84 gene was sequenced and comprises approximately 6 kb of DNA. It is interrupted by 10 intervening sequences. The promoter contains structural features similar to those found in certain housekeeping genes. The genomic sequence of the HSP84 gene from nontransformed parental strain shows no differences in the deduced amino acid sequence compared with Meth A. Six other members of the HSP84 family were characterized and found to be processed pseudogenes having 70-80% homology to HSP84 cDNA. The HSP86 family consists of a structural gene and two highly homologous processed pseudogenes. These genes were assigned to chromosomes 12, 11, and 3. The HSP86 pseudogenes apparently arose more recently than those of the HSP84 family. Comparison of the HSP86 cDNA from the parental and other strains of mice has been performed for nearly the complete molecule. No nucleotide changes that would lead to amino differences have been observed. The manner in which HSP84/86 acts as a TSTA therefore remains an enigma. In light of the recent evidence by Boon and coworkers that tum TSTAs can be expressed as small peptides and still retain their antigenicity, we are exploring the possibility that the tumor-specific nature of the HSP84/86 is due to tumor-specific peptides complexed to these proteins.

We continue our collaborative studies with A. B. DeLeo of the Pittsburgh Cancer Institute in characterizing newly isolated tumor antigens (TATA) from Meth A and other MC-induced sarcomas. A new immunoaffinity-purified antigen, termed 339, has been isolated and studied by tumor rejection and serologic techniques. In contrast to the other known TATA of Meth A, p82 and p86/84, 339 has limited distribution, being found only on thymocytes (as well as Meth A sarcoma cells). SDS-PAGE analysis shows a major 95-kDa band. This protein has been sequenced by conventional methods and has no homology with other presently described TATA and with other known murine differentiation antigens. 339 has strong tumor rejection activity (complete protection) against Meth A, specifically with doses as low as 5  $\mu$ g protein. The monoclonal antibody RH339 recognizes a 95-kDa glycoprotein on the cell surface. A most interesting new characteristic of TATA is revealed in studies of 339. Results clearly indicate 339 to be a functionally active lymphocyte differentiation antigen, in addition to having tumor rejection activity. Work continues to further characterize biochemically and immunologically the gp96 tumor-specific antigen of Meth A. In rather limited



studies the group at Sloan-Kettering had identified the TATA as the glucose-regulating protein gp96. In continuing this work, however, it has now been found that efforts to chromatograph the Con A binding fraction (containing gp96) and isolate gp96 were not reproducible and that the tumor rejection activity could be obtained regularly in a major gp100 fraction that migrates slower and has an  $M_r$  of 100 kDa. None of the gp96 fractions had tumor rejection activity. Further, gp100 fractions regularly induced specific anti-tumor cytotoxic T lymphocyte (CTL) activity, suggesting that tumor rejection and CTL activity are associative properties of chromatographically similar molecules.

Using a group of murine melanomas consisting of the B6 melanomas JB/RH, JB/MS, and B16 F<sub>10</sub> and the C3H/He melanoma K1735, previously we had shown that they share a melanoma-specific TATA and cross-immunize against each other but are antigenically distinct from the DBA/2 melanoma 591. Additionally, this laboratory has shown that the purified gp65 (B700) induces a pattern of cross-immunity identical to that obtained with irradiated cells. B700 is the major protein of the melanosomal membrane. It is a multiple deletion variant of the normally occurring melanosomal protein C700, and cross-reactive molecules have been found in human, swine, and hamster melanomas. To add dimensions to further studies of the significance of B700 and the causes of immunogenicity and specificity, recent studies relate to the production and study of syngeneic antibodies and their role in the immune response and to the production of monoclonal antibodies. We have now defined serologically the TATA of the JB/RH melanoma by C-dependent cytotoxic antibodies present in the sera of syngeneic mice hyperimmunized with the JB/RH melanoma. Absorption tests show that this antigen is also expressed on JB/HS, B16 F<sub>10</sub>, and K1735 membranes but not on the S91 melanoma. These cytotoxic antibodies could be absorbed by the purified B700 glycoprotein. This pattern of expression is identical to that shown in tumor-rejection assays (A. B. DeLeo, V. J. Hearing, W. D. Vieira, and L. W. Law, Serologic detection of a shared melanoma specific antigen of murine melanomas [submitted to *J Immunol*]). Much emphasis has been placed on gangliosides present on human melanoma cells as the source of immunogenicity. JB/RH is one of the few murine melanomas expressing the GM2 ganglioside. It is clear from the above study, however, that the JB/RH antigen we studied is not related to determinants in the glycolipid fraction nor to the GM2 glycolipid purified from this fraction. For a more complete study of the melanoma-specific B700 antigen, monoclonal antibodies are now being produced in studies with Gersten. Those hyperimmunized syngeneic (C57Bl/6) mice testing positive by ELISA for antibodies to B700 were used for preparation of hybridomas. Four positive clones have now been isolated that show specificity to B700 and to melanoma cells.

Two different melanoma-specific monoclonal antibodies in a syngeneic system, show a striking inhibition of the growth of established (5 day) lung metastases of the JB/MS murine melanoma. Doses were as low as 100  $\mu$ g per IV treatment (2 treatments at days 5 and 8 after melanoma cell challenge). Growths subcutaneously, however, were not affected by systemic treatment. Monoclonal antibody 9B6 was induced against B16 F<sub>10</sub> melanoma cells and T97 against B700, the purified melanoma-specific antigen. Both are IgG<sub>2B</sub> isotypes. The B16 F<sub>10</sub> melanoma was less sensitive to monoclonal antibody therapy than JB/MS; however, low doses were effective using 9B6, whereas it has been reported in results from another laboratory that 9B6 was effective only against established liver metastases. Extremely high doses were used (2 mg). This may indicate that anti-metastatic effects may be masked if the system is overwhelmed with large quantities of immunoglobulin. The mechanisms of action of these two monoclonal antibodies are not known. It is clear that a direct cytotoxic or cytostatic mechanism is not responsible for these anti-metastatic effects; preliminary results indicate that natural killer cells function as the host-mediated effector cells. The striking inhibition of established metastatic melanoma growth observed in our studies probably results from the high specificity of our

monoclonal antibodies. Such reagents are difficult to obtain in experimental systems and more difficult for human tumors.

Our laboratory has been involved in characterization of a suppressive immune response. Previous work on the lysozyme-specific CD8<sup>+</sup> T suppressor clone, LH8.105, demonstrated that these cells express the  $\alpha/\beta$  T-cell receptor (TCR) in association with the CD3 complex. The  $\alpha$  and  $\beta$  TCR genes were cloned and sequenced. These cells did not secrete interleukin 2 (IL-2) in response to lysozyme antigen and antigen-presenting cells (APC). However, these cells did produce IL-2 after stimulation with anti-CD3 antibody, 2C11. This lack of IL-2 production to antigen was in contrast to T-helper cells, which produce IL-2 in response to antigen plus APC stimulation. When the turnover of phosphoinositol (PI) in response to antigen was examined, several interesting observations were found with the LH8.105 cells. First, PI turnover can be induced in LH8.105 cells in response to either intact lysozyme or a peptide fragment corresponding to the N terminus of lysozyme. Second, no APC were required to elicit PI turnover; lysozyme alone was able to induce PI turnover, which peaked ~10 min after addition of lysozyme. Finally, the response was antigen specific since neither ovalbumin nor lysozyme peptides, other than that of the N terminus, induced PI turnover. These data demonstrate that LH8.105 can respond to lysozyme apparently without antigen processing and perhaps without the need for MHC presentation. Experiments are underway to show that the recognition of lysozyme by LH8.105 is via the  $\alpha/\beta$  TCR complex.

Interferons (IFNs) play an important role in regulating a variety of host defense mechanisms. Besides up-regulating class I and class II MHC gene products, they exert additional biological effects, including initiation of cell growth and modulation of different immune reactions. The first step in the interaction is the binding of IFN to a specific cell surface receptor. An understanding of this phenomenon will require the molecular characterization of the receptor. We have purified the murine interferon- $\gamma$  ( $\gamma$ -IFN) receptor from a subclone of the EL4 thymoma, which expresses high levels of this receptor. Detergent-solubilized receptor was purified using an anti-receptor antibody under conditions that allowed retention of high-affinity binding activity. The purified receptor was proteolytically digested, individual peptides were purified, and their sequence was determined. The peptides were found to be highly homologous to portions of the human  $\gamma$ -IFN receptor. Nucleotide probes corresponding to the sequence of these peptides are being used to screen a mouse cDNA library to obtain a receptor clone. Isolation of the mouse  $\gamma$ -IFN clone will allow the properties of this receptor to be analyzed in detail upon transfection into various hosts. In addition, these studies will permit the elucidation of the biochemical pathways activated upon receptor occupation, which to date remain poorly defined.

In studies of the regulation of melanogenesis, we have examined the levels of response of mammalian melanocytes to physiologically relevant stimuli, such as ultraviolet light and melanocyte-stimulating hormone. The melanocyte provides a unique model system to study intracellular control mechanisms since only the enzyme tyrosinase is essential for melanin biosynthesis. We have produced tyrosinase-specific monoclonal antibodies which cross-react among mammalian tyrosinases, and have been valuable probes for the characterization of post-translational enzyme processing in melanocytes, and used them to examine the metabolic processing of tyrosinase, which includes glycosylation and intracellular transport to melanosomes, where melanization is initiated. Recently, there have been two different genes cloned which encode tyrosinase, although no primary sequence is available to confirm such an identification. As an approach to unraveling the gene products involved in the regulation of melanogenesis, we have synthesized oligonucleotides encoded by those genes (for hybridization studies) and peptides (used to produce specific antibodies) and

have examined their expression at the transcriptional and translational level in response to environmental stimuli. In agreement with our earlier studies, we have found only slight increases in the synthesis of de novo tyrosinase in response to melanogenic stimulation, and that the dramatic increases in pigmentation result from activation of latent tyrosinase in the melanocyte. We have now cloned a melanoma cell line that produces a large quantity of a melanogenic inhibitor which may be critical to this masking of catalytic activity and have purified and characterized it. This inhibitor is a low molecular weight molecule (~480), probably an amine, which functions in a competitive manner to efficiently inhibit pigment production; it must be produced constitutively to be effective and early results suggest that the effect of melanocyte-stimulating hormone may be to stop inhibitor production, allowing the expression of latent tyrosinase. Aberrations in the regulation of this inhibitory activity may be involved in several types of abnormal pigmentary disorders, such as albinism.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03200-19 LCBGY

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Factors Influencing the Induction, Growth, and Repression of Tumors

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

L. W. Law	Chief	LCB, DCBD, NCI
V. J. Hearing, Jr.	Research Biologist	LCB, DCBD, NCI
K. Kameyama	Visiting Fellow	LCB, DCBD, NCI
W. D. Vieira	Microbiologist	LCB, DCBD, NCI

## COOPERATING UNITS (if any)

Pittsburgh Cancer Institute, Pittsburgh, PA; Georgetown University Medical School, Washington, DC; University of Arizona Health Sciences Center, Tucson, AZ

## LAB/BRANCH

Laboratory of Cell Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2

## OTHER:

1

## 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.)

Type I (restricted) and Type II (cross-reacting) tumor antigens of the transplantation rejection type (TATA), and of tumor antigens (TA) assayed by other *in vivo* and *in vitro* techniques and of the immune responses they evoke have received major emphasis. As a corollary to this study, the biologic properties *in vitro* and *in vivo* of alien histocompatibility (H-2) antigens and of variant antigens in several neoplasms are under study. Purification of TATAs is under investigation with the ultimate purpose of defining these membrane and cytosol antigens, after purification, in physicochemical, biologic, and molecular terms and of assessing their diagnostic and therapeutic potentials.



## Major Findings

### I. Analysis of TATA of MC-induced Sarcomas

#### *p82, p86/84, gp96, and 339 antigens*

We continue our collaborative studies with A. B. DeLeo of the Pittsburgh Cancer Institute in characterizing newly isolated tumor antigens (TATA) from Meth A and other MC-induced sarcomas. A new immunoaffinity-purified antigen, termed 339, has been isolated and studied by tumor rejection and serologic techniques. In contrast to the other known TATA of Meth A, p82 and p86/84, 339 has limited distribution, being found only on thymocytes (as well as Meth A sarcoma cells). SDS-PAGE analysis shows a major 95-kD band. This protein has been sequenced by conventional methods and has no homology with other presently described TATA and with other known murine differentiation antigens. 339 has strong tumor rejection activity (complete protection) against Meth A, specifically with doses as low as 5  $\mu$ g protein. The monoclonal antibody RH339 recognizes a 95-kD glycoprotein on the cell surface. A most interesting new characteristic of TATA is revealed in studies of 339. Results clearly indicate 339 to be a functionally active lymphocyte differentiation antigen, in addition to having tumor rejection activity.

The N-terminal amino acid sequence of antigen 339 is shown; no homology is seen with the other known structures of p82, gp86/84, and gp96.

339 = Ser-Ile-Arg-Val-Leu-Val-Pro-Tyr-X-X-Gly-Val-Leu

Collaborative work continues with DeLeo of Pittsburgh in order to further characterize biochemically and immunologically the gp96 tumor-specific antigen of Meth A. In rather limited studies the group at Sloan-Kettering had identified the TATA as the glucose-regulating protein gp96. In continuing this work, however, it has now been found that efforts to chromatograph the Con A binding fraction (containing gp96) and isolate gp96 were not reproducible and that the tumor rejection activity could be obtained regularly in a major gp100 fraction that migrates slower and has an  $M_r$  of 100 kD. None of the gp96 fractions had tumor rejection activity. Further, gp100 fractions regularly induced specific anti-tumor CTL activity, suggesting that tumor rejection and CTL activity are associative properties of chromatographically similar molecules.

### II. Studies of Murine Melanoma Antigens (TATA)

#### A. Characteristics of Antigens

Using a group of murine melanomas consisting of the B6 melanomas JB/RH, JB/MS, and B16 F<sub>10</sub> and the C3H/He melanoma K1735, previously we had shown that they share a melanoma-specific TATA and cross-immunize against each other but are antigenically distinct from the DBA/2 melanoma 591. Additionally, this laboratory has shown that the purified gp65 (B700) induces a pattern of cross-immunity identical to that obtained with irradiated cells. B700 is the major protein of the melanosomal membrane. It is a multiple deletion variant of the normally occurring melanosomal protein C700, and cross-reactive molecules have been found in human, swine, and hamster melanomas. To add dimensions to further studies of the significance of B700 and the causes of immunogenicity and specificity, recent studies relate to the production and study of syngeneic antibodies and their role in the immune response and to the production of monoclonal antibodies.

## B. Serologic Analyses of Melanoma Antigens

In collaboration with DeLeo, we have now defined serologically the TATA of the JB/RH melanoma by C-dependent cytotoxic antibodies present in the sera of syngeneic mice hyperimmunized with the JB/RH melanoma. Absorption tests show that this antigen is also expressed on JB/HS, B16 F<sub>10</sub>, and K1735 membranes but not on the S91 melanoma. These cytotoxic antibodies could be absorbed by the purified B700 glycoprotein. This pattern of expression is identical to that shown in tumor-rejection assays (A. B. DeLeo, V. J. Hearing, W. D. Vieira, and L. W. Law, Serologic detection of a shared melanoma specific antigen of murine melanomas [submitted to *J Immunol*]).

Much emphasis has been placed on gangliosides present on human melanoma cells as the source of immunogenicity. JB/RH is one of the few murine melanomas expressing the GM2 ganglioside. It is clear from the above study, however, that the JB/RH antigen we studied is not related to determinants in the glycolipid fraction nor to the GM2 glycolipid purified from this fraction.

For a more complete study of the melanoma-specific B700 antigen, monoclonal antibodies are now being produced in studies with Gersten. Those hyperimmunized syngeneic (C57Bl/6) mice testing positive by ELISA for antibodies to B700 were used for preparation of hybridomas. Four positive clones have now been isolated that show specificity to B700 and to melanoma cells.

## C. Melanoma-specific Monoclonal Antibodies Assayed for Therapeutic Efficacy

Using two different melanoma-specific monoclonal antibodies in a syngeneic system, recently we have observed a striking inhibition of the growth of established (5 day) lung metastases of the JB/MS murine melanoma. Doses were as low as 100 µg per IV treatment (2 treatments at days 5 and 8 after melanoma cell challenge). Growths subcutaneously, however, were not affected by systemic treatment. Monoclonal antibody 9B6 was induced against B16 F<sub>10</sub> melanoma cells and T97 against B700, the purified melanoma-specific antigen. Both are IgG<sub>2B</sub> isotypes. The B16 F<sub>10</sub> melanoma was less sensitive to monoclonal antibody therapy than JB/MS, however, low doses were effective using 9B6, whereas it has been reported in results from another laboratory that 9B6 was effective only against established liver metastases. Extremely high doses were used (2 mg). This may indicate that anti-metastatic effects may be masked if the system is overwhelmed with large quantities of immunoglobulin.

The mechanisms of action of these two monoclonal antibodies are not known. It is clear that a direct cytotoxic or cytostatic mechanism is not responsible for these anti-metastatic effects; preliminary results indicate that NK cells function as the host-mediated effector cells.

The striking inhibition of established metastatic melanoma growth observed in our studies probably results from the high specificity of our monoclonal antibodies. Such reagents are difficult to obtain in experimental systems and more difficult for human tumors (see V. J. Hearing, S. P. L. Leong, R. M. Niles, W. D. Vieira, and L. W. Law, Beneficial immunotherapeutic effects of *in vivo* administration of two melanoma-specific monoclonal antibodies in a syngeneic system (submitted to *J Immunol*)).

*Publications*

Gersten DM, Williams LJ, Moody D, Montague PH, Law LW, Hearing VJ. The intracellular association of B700 and B50 murine melanoma antigens and their role in tumor rejection. *Int J Cancer* 1989;43:497-500.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09100-4 LCBGY

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Immunogenicity of Melanoma

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

V. Hearing	LCB, DCBD, NCI
E. Appella	LCB, DCBD, NCI
M. Jiménez	LCB, DCBD, NCI
K. Kameyama	LCB, DCBD, NCI
L. Law	LCB, DCBD, NCI
T. Tsukamoto	LCB, DCBD, NCI

## COOPERATING UNITS (if any)

F. Blasi, Mikrobiologisk Inst., Copenhagen; D. Gersten, Georgetown Univ. Med. Ctr., Washington, DC; A. DeLeo & E. Gorelik, Pittsburgh Cancer Inst., Pittsburgh, PA; S. Leong & J. Bagnara, Univ. of Ariz. Health Sci. Ctr., Tucson, AZ; T. Walden, Armed Forces Radiobiol. Res. Inst., Washington, DC

## LAB/BRANCH

Laboratory of Cell Biology

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4

## PROFESSIONAL:

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## OTHER:

1

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

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project is aimed at characterizing: (1) the host immune response to malignant melanoma, and the role it plays in the progression of tumor growth and metastatic spread; (2) tumor-specific proteins produced by melanoma cells *in vivo* and *in vitro*, to determine the mechanism of their formation, to examine the impact of their expression on tumor growth, and to study the feasibility of utilizing their specificity for the immunoassay and immunotherapy of melanoma; (3) the role of cell surface proteases in the cascade of events leading to metastatic spread of tumors; (4) the control mechanisms involved in the regulation of pigment production in normal and in transformed melanocytes. Our results have demonstrated that various murine melanomas of different origin share a common cell surface antigen which can elicit tumor rejection; this antigen, termed B700, is related biochemically and immunologically to a normal melanocyte protein, perhaps a differentiation antigen. We have found that the immune response elicited by B700 is mediated through cytotoxic antibody production, and not through a demonstrable cellular response; we have now produced syngeneic murine monoclonal antibodies with specific cytotoxicity. The involvement of cell surface urokinase with metastatic potential has been shown by use of specific antibodies which can modulate that potential; we have now isolated melanoma cell clones which vary in their tumorigenic and metastatic properties, and have employed transfection protocols with the genes for urokinase and its receptor to further elucidate the mechanisms involved. We have also further characterized the molecular controls involved in mammalian pigmentation and its activation by environmental stimuli, employing synthetic oligonucleotide and peptide probes to different pigmentation-related gene products. The results have shown that mammalian melanin production is regulated primarily through a post-translational activation of latent enzyme, and that one or more genes normally expressed by melanocytes are repressed in transformed melanocytes, while other melanoma-specific genes are activated following transformation.



## *Objectives*

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.

## *Major Findings*

### *1. Immunogenicity of Malignant Melanoma*

Previously, we (with Law) have shown that murine melanomas, including the K1735, JB/RH, JB/MS, B16, and Harding-Passey, share tumor-specific transplantation antigens (TSTA) which do not cross-react with the S91 melanoma, although the latter produces an S91-specific TSTA. The cross-reactive melanoma TSTA is extremely interesting since the tumors expressing it have arisen over a period of decades as the result of spontaneous events or by chemical- or ultraviolet (UV)-induced transformation. We have shown that B700 (a melanoma tumor-specific antigen, see below), when isolated from the above tumors, can be used to immunize mice and elicit tumor rejection activity, with the specificity noted above. We are engaged in characterizing the mechanisms involved in these responses, and although there is no significant cellular immune response, there is a dramatic humoral response. Our studies (with Law and DeLeo) have shown that mice immunized with melanoma cells produce high titers of cytotoxic antibodies, which can be quantitatively absorbed with the B700 antigen; these have now been used to produce monoclonal antibodies which have the requisite specificity and cytotoxicity. The specificity of this cytotoxicity is identical to the pattern of cross-reactive TSTA activity noted above, suggesting that the major immune response of mice to challenge with melanoma cells is the production of cytotoxic antibodies and not the generation of cell-mediated immune mechanisms. Since these responses are rarely effective in preventing tumor growth in naive animals, our current studies are directed at examining the reasons behind this ineffective response, and at ways to successfully augment it. *In vivo*, B700 has been shown to associate with another melanoma-specific antigen, termed B50, although the role this heterocomplex might play in the expression of cell surface TSTA, or in the sensitivity of melanoma cells to immune response mechanisms, has not yet been elucidated. In addition, another C57B16 melanoma-specific antigen, termed 9B6, has now been shown to be associated with the expression of a melanoma-specific, ecotropic murine retrovirus; the mechanism whereby the expression of this virus is limited to melanomas arising only from C57B16 mice is an exciting question now being addressed in collaborative studies. Monoclonal antibodies recognizing the 9B6 antigen or the B700 antigen have now been successfully used to decrease the proliferation of established pulmonary metastases of melanoma cells, presumably through their stimulatory action on natural killer (NK) cell activity.

### *2. Tumor-Specific Antigens Produced by Malignant Melanoma*

Transformed cells express dramatically different cellular products than do their normal counterparts; the production of novel products (TSA) by malignant cells

(i.e., those not produced by normal cells) and the involvement of TSA in the growth and function of tumor cells has been a subject of widespread interest. We (with Gersten) have examined two such TSA, termed B700 and B50. Both of these antigens seem to be analogous to normal melanocyte proteins, but with alterations in their primary structure which gives rise to their immunologic specificity. The ability of B700 to also function as a TSTA in cells which express it on the cell membrane surface has recently been shown by our group (see above). We have also found that B700, or a cross-reactive antigen, is expressed specifically by melanomas of human, hamster, and swine origin, in addition to the murine melanomas. We have now produced murine monoclonal antibodies specific to B700, and are collaborating on a project to clone its gene; such information should allow us to characterize the mechanism(s) whereby this TSA is expressed by transformed melanocytes, but not by normal melanocytes. In collaborative efforts (with Gersten and Walden) we have begun a functional analysis of B700 action, showing it has an altered catalytic activity on prostaglandin E2 compared to serum albumin. We (with Leong and Gorelik) have also examined the specificity of another melanoma-specific antigen, termed 9B6, which was identified by syngeneic monoclonal antibodies produced against B16 melanoma cells. Our studies have shown that this antigen is expressed only by melanomas arising in C57Bl mice (either spontaneous or chemically induced); interestingly, expression of the antigen is dependent on the replication and budding of a B-tropic ecotropic murine retrovirus. Whether the antigen is encoded by the virus, or merely induced to express specifically by melanocytes producing the virus, is currently being studied; the specificity of this antigen with C57Bl-derived melanomas is an interesting phenomenon which is also under investigation.

### 3. *Metastatic Events in Dissemination of Malignant Melanoma*

We (with Appella, Blasi, and Law) have examined the role of cell surface membrane expression of proteases on tumor invasion and metastasis. We have shown that urokinase, a protease involved in the control of migration and differentiation of normal cells, is critical to the tumor cell's capability to penetrate membranes and other proteinaceous barriers during metastasis of transformed cells. We have shown that alteration of surface urokinase activity by immunological and enzymatic reagents has dramatic consequences on the metastatic behavior of melanoma cells, which correlates directly with increases or decreases in urokinase catalytic activity. We have addressed the question of whether this parameter is important *in vivo* by cloning B16 and JB/MS murine melanoma cells, using no selection pressure, then examining the metastatic potentials and the urokinase expression of the cloned lines; again, there was a correlation between metastatic activity and surface localization of urokinase activity. These studies suggest that urokinase is an important, although not a sufficient, parameter in the determination of a tumor cell to metastasize. We have succeeded in transfecting the urokinase gene into poorly metastatic cells in order to directly determine the effect of enhanced urokinase expression on metastatic behavior; to date those cells have not shown enhanced metastatic potential compared to mock transfected cells. In recognition of the importance of the surface receptor for localization of urokinase at the cell surface, we have suggested that the receptor may be the more important molecule in determining a cell's ability to proteolytically degrade biological membranes. The uPA receptor has now been cloned, and we are in the process of a collaborative effort aimed at examining the effect of the uPA receptor on metastatic potential, using transfection techniques. We have also examined the relationship of differentiation with the tumorigenic and metastatic phenotypes of melanoma cells. Using melanocyte-stimulating hormone (MSH), we (with Law, Kameyama, and Tsukamoto) have characterized the effect of MSH-induced differentiation on those properties

of melanoma cells, either with pretreatment prior to challenge or with treatment of animals post-challenge. While induced differentiation inversely affects tumorigenic abilities, i.e., the capacity to grow as primary subcutaneous tumors, it has different effects on metastatic potential (in most cases causing increases in metastatic growth). These findings are in agreement with studies examining the expression and involvement of uPA with differentiation; future studies will be directed at examining melanoma cell characteristics modulated by MSH treatment that are critical to tumorigenic and metastatic potentials.

#### 4. Regulation of Melanogenesis

We (with Jiménez, Kameyama, and Tsukamoto) have examined the levels of response of mammalian melanocytes to physiologically relevant stimuli, such as UV light and MSH. The melanocyte provides a unique model system to study intracellular control mechanisms since only the enzyme tyrosinase is essential for melanin biosynthesis, although the rates of pigmentation can be influenced at various levels. We have produced tyrosinase-specific monoclonal antibodies which cross-react among mammalian tyrosinases, and have been valuable probes for the characterization of post-translational enzyme processing in melanocytes. We have used them to examine the metabolic processing of tyrosinase, which includes glycosylation and intracellular transport to melanosomes, where melanization is initiated. Recently, there have been two different genes cloned which encode tyrosinase, although no primary sequence is available to confirm such an identification. As an approach to unraveling the gene products involved in the regulation of melanogenesis, we have synthesized oligonucleotides encoded by those genes (for hybridization studies) and peptides (used to produce specific antibodies) and have examined their expression at the transcriptional and translational level in response to environmental stimuli. In agreement with our earlier studies, we have found only slight increases in the synthesis of *de novo* tyrosinase in response to melanogenic stimulation, and that the dramatic increases in pigmentation result from activation of latent tyrosinase in the melanocyte. We have now cloned a melanoma cell line that produces a large quantity of a melanogenic inhibitor which may be critical to this masking of catalytic activity and have now purified and characterized it. This inhibitor is a low molecular weight molecule (~480), probably an amine, which functions in a competitive manner to efficiently inhibit pigment production; it must be produced constitutively to be effective and early results suggest that the effect of MSH may be to stop inhibitor production, allowing the expression of latent tyrosinase. Aberrations in the regulation of this inhibitory activity may be involved in several types of abnormal pigmentary disorders, such as albinism. In addition, we have shown that levels of melanogenesis correlate closely with the expression of surface MSH receptors, which in turn can be regulated by various environmental stimuli, such as cytokines (e.g., interferon), UV light, and MSH itself (as an autocrine function). The emerging picture indicates that the control of mammalian pigmentation is an order of magnitude more complex than originally thought, but recent advances at the molecular level are slowly unraveling these interrelated control mechanisms, many of which may be malfunctioning in various pigmentary disorders.



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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03229-19 LCBGY

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## TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

T-Cell Antigen Recognition and Tumor Antigens

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

E. Appella	Medical Officer	LCB, DCBD, NCI
F. Cofano	Guest Worker	LCB, DCBD, NCI
S. K. Moore	Senior Staff Fellow	LCB, DCBD, NCI
J. W. Romano	IRTA Fellow	LCB, DCBD, NCI
S. Tanaka	Visiting Fellow	LCB, DCBD, NCI
S. J. Ullrich	Senior Staff Fellow	LCB, DCBD, NCI
J. Ashwell	Medical Officer	BRMP, DCT, NCI
R. Hodes	Medical Officer	EIB, DCBD, NCI
K. Ozato	Research Microbiologist	LDMI, NICHD

## COOPERATING UNITS (if any)

Cytel, La Jolla, CA; Preclinical Research, Sandoz Ltd., Basel, Switzerland;  
 Mikrobiologisk Institute, Copenhagen, Denmark; A.R.C., Villejuif, France

## LAB/BRANCH

Laboratory of Cell Biology

## SECTION

Chemistry

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

6

## PROFESSIONAL:

6

## OTHER:

0

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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.)

Our research involves the binding of antigens to class II major histocompatibility antigens (MHC) and T-cell helper and suppressor antigen receptors, the structure of helper and suppressor factors, MHC transcriptional activators, and tumor antigens. Synthetic peptides were used to determine the general consensus sequence necessary for peptide binding to class II MHC I-A<sup>d</sup> and I-E<sup>d</sup> molecules. The consensus sequence was used to predict the ability of potential antigens to bind to these molecules. A transcriptional factor that binds to the regulatory element of the MHC class I gene was cloned and found to belong to the superfamily of nuclear steroid hormone receptors. Analysis of conditioned media from T-helper and -suppressor cells revealed the presence of a "soluble" T-cell receptor/CD3 complex.

The sequences of Meth A tumor-specific transplantation antigens (TSTA), previously identified as the 84- and 86-kDa heat shock proteins (HSP), have been completed. The sequence of the normal counterpart is almost complete for the 86-kDa HSP, and no differences have been noted. The antigenic nature of these proteins is currently being investigated with the emphasis on peptide antigens associated with these proteins. Another TSTA from a xenogenized tumor was found to be the endogenous xenotropic retroviral protein, gp70, and was found to elicit IgG antibody and CTL responses. The sequence of the p53 oncogene from a human osteosarcoma was determined and found to have a point mutation leading to a single amino acid change compared to the normal protein.

The murine IFN- $\gamma$  receptor has been purified and its amino acid sequence partially determined, and these are being used to synthesize probes to be used to screen a cDNA library. The sequence of urokinase receptor has also been determined.

## Major Findings

### *T-Cell Antigen Recognition and Major Histocompatibility Antigens*

T-cells recognize protein antigens on the surface of antigen-presenting cells (APC) only after the antigen has been appropriately processed by the APC, associated with class II major histocompatibility complex (MHC) molecules, and subsequently displayed on the cell surface. Several studies on the mechanism of antigen recognition by T-cells have established that recognition requires a complex between MHC and peptide fragments of the protein antigen. Our main interest during the last year was to gain insights on the structural basis of the interaction between peptides and MHC molecules. We were specifically interested in why the few MHC molecules expressed by an individual can bind a nearly limitless number of antigenic peptides. We were further interested in the relevance of this determinant selection by MHC molecules in defining immune responses to protein antigens. To understand how a large universe of immunogenic peptides can bind to and be restricted by a very small number of class II MHC molecules (Ia) present within an individual, we have undertaken to characterize the requirements for the binding of peptides to a given Ia molecule. These studies were carried out utilizing the class II MHC-restricted molecule, I-E<sup>d</sup>, and a set of peptides that were capable of binding to the I-E<sup>d</sup> molecule. To define the region of I-E<sup>d</sup> that interacts with peptide antigen, our work has concentrated on the immunogenic peptide from hen egg lysozyme (HEL) 105-120. By synthesizing a series of N- and C-terminal truncated analogs and testing these for binding to purified I-E<sup>d</sup> molecules, we were able to define a critical core region within HEL 105-120 that consisted of the heptapeptide contained in the region HEL 110-116. This core region can be identified in several unrelated peptides that bind to the I-E<sup>d</sup> molecule. A common motif of multiple basic amino acid residues determines the binding capacity of the peptide to the I-E<sup>d</sup> molecule. We have also analyzed a large panel of single amino acid substitution analogs of HEL 107-116. Significant effects on I-E<sup>d</sup> binding were seen only when non-conservative substitutions at one of the three basic residues were made. These results confirm the very permissive nature of peptide binding to Ia, in that 90% of the substitutions had little or no effect on the binding capacity of HEL 107-116. The specificity of the immune response is maintained by the specificity of T-cell recognition. Twenty-five of thirty amino acid substitutions analyzed resulted in more than a 10-fold reduction in antigenicity for two T-cell hybridomas specific for the I-E<sup>d</sup>-restricted peptide HEL 107-116. Another interesting point is the finding that the degeneracy of the interaction between Ia and peptides allows class II molecules to bind many structurally unrelated peptides through the same binding site. Recognition of such unrelated peptides is, however, accomplished by recognition of structurally "broad" motifs as identified for both I-E<sup>d</sup> and I-A<sup>d</sup>. Such motifs could be used to predict peptides that are strong binders to I-A<sup>d</sup> or I-E<sup>d</sup>. The accuracy of these predictions has been tested by a computer program on a large panel of synthetic peptides and a set of overlapping peptides from the staphylococcal nuclease protein. Our results from actual binding studies indicate that it is possible to predict successfully about 75% of the strong I-A<sup>d</sup> binding peptides. This suggests that definition of such sequence "motifs" could be of general use in predicting potentially immunogenic peptide regions within proteins.

Our laboratory has also been involved in characterization of the suppressive immune response. Previous work in our laboratory on the lysozyme-specific CD8<sup>+</sup> T suppressor clone, LH8.105, demonstrated that these cells express the  $\alpha/\beta$  T-cell receptor (TCR) in association with the CD3 complex. The  $\alpha$  and  $\beta$  TCR genes were cloned and sequenced. These cells did not secrete interleukin 2 (IL-2) in response to lysozyme antigen and APC. However, these cells did produce IL-2 after stimulation with anti-CD3 antibody, 2C11. This lack of IL-2 production to antigen

was in contrast to T-helper cells, which produce IL-2 in response to antigen plus APC stimulation. When the turnover of phosphoinositol (PI) in response to antigen was examined, several interesting observations were found with the LH8.105 cells. First, PI turnover can be induced in LH8.105 cells in response to either intact lysozyme or a peptide fragment corresponding to the N terminus of lysozyme. Second, no APC were required to elicit PI turnover; lysozyme alone was able to induce PI turnover, which peaked ~10 min after addition of lysozyme. Finally, the response was antigen specific since neither ovalbumin nor lysozyme peptides, other than that of the N terminus, induced PI turnover. These data demonstrate that LH8.105 can respond to lysozyme apparently without antigen processing and perhaps without the need for MHC presentation. Experiments are underway to show that the recognition of lysozyme by LH8.105 is via the  $\alpha/\beta$  TCR complex.

T cells and B cells can be positively or negatively regulated by soluble factors produced by T-helper ( $T_h$ ) or T-suppressor ( $T_s$ ) cells, respectively. These factors,  $T_HF$  and  $T_SF$ , are antigen specific and H-2 restricted but have not been well characterized biochemically due to their high potency and low level of synthesis.  $T_HF$  was analyzed from cloned antigen-specific and MHC-restricted type 2  $T_h$  cells whose culture supernatants support trinitrophenyl (TNP)-specific IgG antibody response in TNP-primed B cells. An anti-T-cell receptor monoclonal antibody, F23.1, specific for  $V_{\beta}8$  TCR was found to remove a substantial amount of the  $T_h$  activity of culture supernatant from F23.1<sup>+</sup>  $T_h$  clones but not from F23.1<sup>-</sup>  $T_h$  clones. Immunoprecipitation analysis of metabolically labeled F23.1<sup>+</sup>  $T_h$  clones revealed the presence of a "soluble" TCR/CD3 receptor complex in conditioned culture supernatants that was indistinguishable from that present at the cell surface. Moreover, gel filtration analysis and ultracentrifugation analysis of the conditioned media indicate that the TCR/CD3 complex probably represents shed membrane vesicles that are highly enriched for the TCR complex as evidenced by the lack of other cell surface proteins such as MHC class I molecules. These data strongly suggest that the  $T_HF$  is composed of the TCR/CD3 complex in association with membrane lipid. Thus, antigen specificity of  $T_HF$  is apparently derived from the unique specificity of the helper clone's own TCR and not via different antigen-specific molecule(s). Analysis of the conditioned culture media of the LH8.105 suppressor cell clones revealed the presence of  $\alpha/\beta$  TCR that was shed from the cell surface, similar to that found in  $T_h$  cell conditioned media. As found with the  $T_h$  cells, it is likely that the soluble receptor may be the antigen-specific  $T_SF$ , and experiments are in progress to test this possibility.

#### *Tumor-Specific Transplantation Antigens*

The 3-methylcholanthrene-induced murine fibrosarcoma, Meth A, has been shown to display at least three different tumor-specific transplantation antigens (TSTA). One of the TSTA is an 82-kDa cytosolic protein that is also present in non-transformed cells. Another of the TSTA was identified as the 96-kDa glucose-regulated protein. The third TSTA is a mixture of two heat shock proteins, designated HSP84 and HSP86. These latter proteins have been studied extensively in this laboratory. HSP84 and HSP86 are coded for by separate, but related, genes. Full-length cDNA clones have been isolated and their nucleotide sequences determined. The deduced protein sequences of HSP84 and HSP86 are 724 and 733 amino acids residues, respectively. The two proteins are 86% homologous, whereas their cDNA coding regions are 76% homologous. The genes coding for these proteins apparently arose by a gene duplication some 500 million years ago. HSP-related sequences comprise a family of genes, three of which were assigned to chromosomes 17, 12, and 2. The HSP structural gene has been mapped to 5.6 cM telomeric to the MHC class I region on chromosome 17. The HSP84 gene was sequenced and comprises approximately 6 kb of DNA. It is interrupted by 10 intervening sequences. The



promoter contains structural features similar to those found in certain housekeeping genes. The genomic sequence of the HSP84 gene from nontransformed parental strain shows no differences in the deduced amino acid sequence compared with Meth A. Six other members of the HSP84 family were characterized and found to be processed pseudogenes having 70-80% homology to HSP84 cDNA. The HSP86 family consists of a structural gene and two highly homologous processed pseudogenes. These genes were assigned to chromosomes 12, 11, and 3. The HSP86 pseudogenes apparently arose more recently than those of the HSP84 family. Comparison of the HSP86 cDNA from the parental and other strains of mice has been performed for nearly the complete molecule. No nucleotide changes that would lead to amino differences have been observed. The manner in which HSP84/86 acts as a TSTA therefore remains an enigma. In light of the recent evidence by Boon and coworkers that tum<sup>r</sup> TSTAs can be expressed as small peptides and still retain their antigenicity, we are exploring the possibility that the tumor-specific nature of the HSP84/86 is due to tumor-specific peptides complexed to these proteins. Another aspect currently being tested is the ability of these antigens to be recognized by T-cells employing  $\gamma/\delta$  chains of the TCR, since this subset of T-cells has recently been implicated in the recognition of HSPs.

We have recently characterized another TSTA from the chemically xenogenized tumor L5178Y/DITC. Syngeneic sera from mice bearing these tumors are able to immunoprecipitate an ~80-kDa cell surface glycoprotein, which was shown to be the endogenous retroviral protein, gp70, of the xenotropic class. Mice specifically immunized with the ~80-kDa antigen developed specific immunity to this tumor in terms of increased IgG antibody response and frequency of cytotoxic lymphocyte precursors to the xenogenized tumor. These CTLs were specifically blocked by the anti-80-kDa sera. Polymerase chain reaction analysis of the tumor and the non-immunogenic parental cells should allow definition of the nature of the change(s) in the gene that renders it immunogenic.

#### *Transcriptional Activators of Class I MHC Antigens*

MHC class I gene expression is controlled by two *cis*-regulated sequences: a conserved regulatory element (CRE) that resides between nucleotides -203 and -161 and the interferon consensus sequence (ICS) that resides between nucleotides -167 and -139. Within the MHC CRE there are three regions (I, II, and III) that bind individual nuclear factors. Factors that bind to regions I and II are present at various times and correlate with development and tissue-specific class I expression. To isolate genes encoding these factors we have screened an unamplified lambda gt11 mouse liver cDNA library with <sup>32</sup>P-labeled concatenated CRE and ICS probes. A cDNA clone that encodes a protein capable of binding to region II of the CRE, but not to the ICS, was isolated. The DNA sequence of the isolated clone revealed that this factor is a member of the superfamily of nuclear hormone receptors. The most interesting feature of the superimposed sequence is the presence of two zinc fingers, each containing four or five cysteines. This structure is typical of steroid or thyroid hormone receptors. We have also found that sequences homologous to that of the above factor are conserved in turkey, chicken, *Xenopus*, and *Drosophila* DNA. Furthermore, the RNA from these genes is expressed at many different times during development.

Interferon (IFN) induces transcription of class I genes through interaction at the ICS. We have obtained data that indicate the ICS binds a constitutive nuclear factor and two inducible nuclear proteins. Recently, we have screened a lambda gt11 macrophage cDNA library with a <sup>32</sup>P-labeled, concatenated ICS probe and obtained a clone that encodes one of the inducible factors. The DNA sequence of the clone is being determined.



IFNs play an important role in regulating a variety of host defense mechanisms. Besides up-regulating class I and class II MHC gene products, they exert additional biological effects, including initiation of cell growth and modulation of different immune reactions. The first step in the interaction is the binding of IFN to a specific cell surface receptor. An understanding of this phenomenon will require the molecular characterization of the receptor. We have purified the murine interferon- $\gamma$  ( $\gamma$ -IFN) receptor from a subclone of the EL4 thymoma, which expresses high levels of this receptor. Detergent-solubilized receptor was purified using an anti-receptor antibody under conditions that allowed retention of high-affinity binding activity. The purified receptor was proteolytically digested, individual peptides were purified, and their sequence was determined. The peptides were found to be highly homologous to portions of the human  $\gamma$ -IFN receptor. Nucleotide probes corresponding to the sequence of these peptides are being used to screen a mouse cDNA library to obtain a receptor clone. Isolation of the mouse  $\gamma$ -IFN clone will allow the properties of this receptor to be analyzed in detail upon transfection into various hosts. In addition, these studies will permit the elucidation of the biochemical pathways activated upon receptor occupation, which to date remain poorly defined.

#### *Protein Chemistry*

A protein chemoattractant for neutrophils, interleukin 8, has recently been defined, having sequence identity to several host defense cytokines. All members of this group of proteins contain four half-cysteine residues at identical positions. The solution conformation has been investigated by two-dimensional nuclear magnetic resonance spectroscopy. The protein contains a triple-stranded anti-parallel  $\beta$ -sheet and a long C-terminal helix. The determination of the three-dimensional structure in solution is currently in progress. A second protein chemoattractant for human monocytes has been purified from the culture supernatant of a glioma cell line and phytohemagglutinin-stimulated human mononuclear leukocytes. This monocyte chemoattractant is part of another group of proteins with four half-cysteine residues. They can be distinguished from the first group, since the first two half-cysteines are adjacent. Many mitogenic and activation stimuli cause secretion of the monocyte attractant by a wide variety of cells, including lymphocytes and vascular smooth muscle cells. Detailed analysis of the three-dimensional structure is being carried out, a key to understanding the structure/function relationship of these proteins.

The surface receptor for urokinase plasminogen activator (uPA) has been recognized in recent years as a key molecule on regulating plasminogen-mediated extracellular proteolysis. The presence of receptors on the cell surface for plasminogen and uPA provides the cells with the potential to produce surface-bound plasmin. This property regulates intercellular adhesions, degradation of basement membranes and the extracellular matrix, and therefore the capacity of the cells to migrate and invade neighboring tissues. We have biochemically purified the uPA receptor and obtained a partial N-terminal sequence. An oligonucleotide probe synthesized on the basis of the N-terminal sequence has been used to screen a cDNA library. Several clones were obtained, and the sequence of one of these appears to encode the complete uPA receptor molecule. The availability of the uPA receptor cDNA will allow a detailed study of its interaction with ligand, as well as of its role in invasive and migratory abilities of malignant and embryonic cells, respectively.

*Isolation and Biological Significance of a Mutated p53 Gene*

The nuclear phosphoprotein p53 is a critical component of the normal cell cycle, regulating the transition of cells from G<sub>1</sub> into S phase. Mutant forms of p53, however, have been shown to cooperate with *ras* in the transformation of rat embryo fibroblasts. Despite this oncogenic potential of mutant p53, wild-type p53 has been shown to suppress the cooperation between *ras* and mutant p53. This suggests that normal p53 may suppress the immortalized phenotype of transformed cells. Mutant p53 in the mouse had also been shown to form tight complexes with members of the 70-kDa family of HSPs. We recently established the first association of human p53 with the hsp 72/hsc 73 HSPs, in the human osteosarcoma (HOS)-derived cell line HOS-SL. Given this association, we wanted to determine if in fact a mutant form of p53 was encoded by these cells. We constructed a cDNA library from the HOS-SL cells and obtained two independent and distinct cDNA clones. These clones were sequenced and shown to be identical at all comparable bases. They were also identical to all previously determined human p53 sequences, except at codon 156, where a G to C point mutation in the HOS-SL sequence led to an arginine to proline amino acid substitution. This apparent mutation in p53 is the first identification of such an event in osteosarcoma cells.

Several phenotypically different sublines have been derived by various treatments of the nontransformed, parental HOS osteosarcoma cell line. These include both virally and chemically transformed sublines, as well as revertant sublines. The polymerase chain reaction was utilized to amplify genomic DNA from these cell lines over the region containing the codon 156 mutation. Oligonucleotide probes, specific for the wild-type and mutant sequences, were used in differential hybridization analysis of these amplified DNAs to determine what sequence was present at codon 156. In each of the cell lines assayed by this method, only the mutant sequence could be detected. Importantly, the cell line KHOS/NP, which is a tumorigenic subline transformed by the Kirsten mouse sarcoma virus, contained the mutant p53 sequence. Since Ki-MSV contains the *v-ras* gene, the fact that these cells are fully transformed (by various growth and tumorigenesis assays) suggests that the type of cooperation between mutant p53 and *ras* observed in the mouse is also possible in human cells.

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## Annual Report

### LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY FY 1989

The Laboratory of Tumor Immunology and Biology carries out a range of investigations in tumor immunology, tumor cell biology, and molecular biology. Areas of particular emphasis include: (a) the identification and characterization of cellular genes associated with the development of carcinoma, both in rodents and humans; (b) biochemical mechanisms of oncogene expression, and cell growth regulation; and (c) the characterization and utilization of monoclonal antibodies directed against tumor-associated antigens, with emphasis being placed on the study of human carcinoma cell populations, and potential diagnostic and therapeutic applications.

The Laboratory of Tumor Immunology and Biology is composed of five 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. Yoon Sang Cho-Chung, Chief). Although each of the five Sections represents an independent program of scientific research, many collaborative studies are carried out between Sections. Below is a summary of the research activities being carried out in the Laboratory of Tumor Immunology and Biology.

#### BIOCHEMISTRY OF ONCOGENES SECTION (R. Bassin, Chief)

"Anti-oncogenes": The Analysis of Cellular Resistance to Transformation. A new technique has been developed for isolating cells that resist transformation by specific oncogenes using 4-*cis*-hydroxy-L-proline as a selective agent. Additional revertants are being characterized from *ras*-transformed cells. The new revertants are resistant to transformation by members of the *ras* gene family and to certain tyrosine kinase oncogenes, but they are sensitive to retransformation by *mos*, *raf*, and *fos*. Combining the retransformation data observed here with those from our ouabain-derived revertants isolated previously (C-11 and F-2), it has been found that all oncogenes can be placed into one of four groups, and data suggests that this represents a primitive functional classification. If true, the *ras* gene would require the role of at least two cellular functions (such as attachment to the cell membrane and kinase activity) to successfully transform mouse cells.

In another series of experiments, 3 of 10 otherwise inactive human tumor DNA samples tested positive for focus-forming activity when used to transfect indicator cells incubated without serum. Cells infected with the mouse *c-myc* gene in a retroviral vector also efficiently transform NIH/3T3 clone 7 cells in the absence of serum. The inhibition of focus-forming activity of *c-myc* appears to take place after the synthesis of *myc*-specific mRNA. The nature of the inhibitor present in serum is now being studied.

The Role of TGF $\alpha$  in the Etiology and Progression of Breast Cancer. Transforming growth factor alpha (TGF $\alpha$ ) has been circumstantially implicated in the autocrine growth of a number of different rodent and human tumor cells *in vitro*. However, its distribution and role in the etiology and/or progression of rodent and human breast cancer are relatively unknown. The present studies have demonstrated that biologically active and immunoreactive TGF $\alpha$  can be detected in hormone (estrogen)-dependent, DMBA- or NMU-induced rat mammary adenocarcinomas and that these tumors possess a specific 4.8-kb TGF $\alpha$  mRNA. These tumors also express elevated levels of c-Ha-*ras* protein (DMBA tumors) or possess a point-mutated c-Ha-*ras* gene (NMU tumors). Ovariectomy results in tumor regression and is

preceded by a specific decrease in TGF $\alpha$  mRNA and protein. Transformation of nontransformed mouse mammary epithelial cell lines with a point-mutated c-Ha-ras protooncogene results in the loss in mitogenic responsiveness of these cells to exogenous epidermal growth factor (EGF) and a reduction in the number of unoccupied EGF receptors on these cells due in part to an enhanced production and secretion of TGF $\alpha$ . Several human breast cancer cell lines also secrete TGF $\alpha$  and possess TGF $\alpha$  mRNA. In the estrogen-responsive breast cancer cell lines, estrogen can induce a 3- to 5-fold increase in TGF $\alpha$  mRNA and protein. Treatment of these cells with a polyclonal anti-TGF $\alpha$  antibody or with a monoclonal anti-EGF receptor antibody can inhibit the growth of these cells *in vitro*. Elevated levels of TGF $\alpha$  protein or expression of TGF $\alpha$  mRNA can be detected in 50-60% of primary human breast tumors. No evidence was found for any gross amplifications and/or rearrangements of the TGF $\alpha$  gene in these tumors. Additionally, overexpression of a human TGF $\alpha$  gene in a cloned immortalized population of mouse mammary epithelial cells can lead to the malignant transformation of these cells *in vitro* and *in vivo*. Collectively, these results suggest that TGF $\alpha$  can function as an autocrine growth factor for a subset of rodent and human breast tumors, that estrogens or activated protooncogenes such as ras can enhance the expression of TGF $\alpha$ , and that enhanced production of TGF $\alpha$  can lead to the transformation of mammary epithelial cells that have a sufficient complement of functional EGF receptors.

#### CELLULAR BIOCHEMISTRY SECTION (Y. S. Cho-Chung, Chief)

The Cellular Biochemistry Section studies the control mechanism of cell growth and differentiation by cyclic AMP (cAMP). Using new derivatives of cAMP, site-selective cAMP analogs, whose effects far exceed that of parent cAMP or the previously studied cAMP analogs, we anticipate achieving our research goals in the following areas: (1) a better understanding of the regulatory mechanisms of cell growth and differentiation, (2) a clearer definition of the derangement of cAMP-effector function in neoplastic transformation and progression, and (3) improved management of human cancers by providing the non-toxic biological compounds (site-selective cAMP analogs) as antineoplastics and chemopreventives. Our studies being conducted in two projects are summarized below.

Site-selective cAMP Analogs as Antineoplastics and Chemopreventives. The physiologic role of cAMP in the growth control of a spectrum of human cancer lines, including leukemic lines, v-rasH oncogene-transformed NIH/3T3 cells, and v-ras<sup>K1</sup> or TGF $\alpha$ -transformed NRK (normal rat kidney) cells, is demonstrated by the use of site-selective cAMP analogs. These cAMP analogs, which can select either of the two known cAMP binding sites of the cAMP receptor protein, induce potent growth inhibition, phenotypic change, and differentiation of cancer cells (leukemic cells) at micromolar concentrations with no sign of cytotoxicity. The growth inhibition parallels selective modulation of cAMP-dependent protein kinase isozymes, type I versus type II, and suppression of cellular proto-oncogene expression. Site-selective cAMP analogs thus provide new biological tools for investigating cell proliferation and differentiation and also for the improved management of human cancers.

Mechanism of cAMP Action in Growth Control and Differentiation: Gene Regulation. Recent evidence suggests a direct action of cAMP on mammalian genes in which the cAMP-dependent protein kinase might interact directly with target genes. Since cAMP-dependent protein kinase is exclusively present in the cytoplasm, any nuclear function of the protein kinase must require its nuclear translocation. It was found that the growth inhibition and morphological change (human cancer cell lines), differentiation (leukemic cells), and phenotypic reversion (v-ras<sup>H</sup>-, v-ras<sup>K1</sup>-, and TGF $\alpha$ -transformed cells) induced by site-selective cAMP analogs correlated with the nuclear translocation of RII cAMP receptor protein, the regulatory subunit of protein kinase type II, which was detected within 10 min after the analog treatment. The goal of this study is to provide direct evidence



of interaction between the cAMP consensus sequences, cAMP responsive element (CRE) and cAMP receptor protein and/or a phosphoprotein substrate of protein kinase.

#### ONCOGENETICS SECTION (R. Callahan, Chief)

Mammary Tumorigenesis in Inbred and Feral Mice. The study of experimentally induced mammary tumors has focused primarily on several mouse strains that are infected with mouse mammary tumor virus (MMTV) and have been inbred for a high incidence of mammary tumors. We have expanded this study to include other species of MMTV-infected feral mouse strains that have not been bred for high mammary tumor incidence. MMTV appears to induce mammary tumors by acting as an insertional mutagen that leads to the activation of previously silent cellular genes (*int* genes). We have previously shown that the inbreeding program used to develop the high-incidence mouse strains may also have fixed other cellular mutations that affect the frequency with which *int-1* and *int-2* are activated. The *int-1* and *int-2* genes are much less frequently activated in feral mouse mammary tumors, raising the possibility that other *int* loci are active in these tumors. We described one such locus, designated *int-3*, in CZECHII V<sup>+</sup> feral mice. The organization of *int-3* differs from that of *int-1* or *int-2*. MMTV insertions occur within a 500-bp region of the cellular genome on chromosome 17. Viral insertion at this locus leads to the activation of the expression of two cellular RNA species that correspond to host flanking sequences located 5' and 3' of the viral insertion site, respectively. We are currently determining the nucleotide sequence of recombinant cDNA clones corresponding to each of these RNA species. Mice chronically infected with MMTV frequently contain preneoplastic hyperplastic alveolar nodules (HAN), which can be transplanted as hyperplastic outgrowth (HOG) lines. We have found that in three of four CZECHII HOG lines, *int-1* is occupied by an MMTV genome. This suggests that activation of *int-1* is an early event in MMTV-induced mammary tumorigenesis. Human breast tumors frequently express tumor-associated antigens. One of these, the carcinoembryonic antigen (CEA) is a member of the family of related glycoproteins that are expressed normally in fetal tissue, the placenta, and other adult tissues. We have found that MMTV-induced CZECHII V<sup>+</sup> mammary tumors express a 4.0-kb RNA species that is related to human CEA.

Biological Effect(s) of Oncogenetic Elements on Mammary Cell Growth and Senescence. A number of genetic elements have been implicated in the process of malignant transformation in the mammary glands of experimental animals and humans. The mouse mammary gland provides an excellent experimental model within which to elucidate the function of these putative mammary oncogenes during the growth, development, and differentiation of mammary tissue *in vivo*. Any portion of the adult mouse mammary gland at any age and throughout all stages of differentiation can repopulate the gland-free mammary fat pad of syngeneic mice upon transplantation. Similar results are obtained when dissociated glandular epithelium is injected into the fat pad or alternatively grown in culture under prescribed conditions prior to reintroduction into the mammary fat pad. We have initiated experiments designed to evaluate the feasibility of introducing functional genetic elements into mouse mammary cells with recombinant retroviral expression vectors antecedent to the reintroduction of these cells into a gland-free fat pad. Paramount to the success of such a project is the maintenance of the "repopulation potential" of the mammary epithelial cells. Our studies and those of others indicate that the number of cells capable of repopulation of the fat pad is small initially and the ability to repopulate is gradually lost through mitotic events. We have morphologically identified a potential candidate for these "mammagenic" cells *in situ* and in explant cultures. These cells seem to differentially express certain cyokeratins during growth and expansion of the mammary gland *in vivo*, suggesting that specific keratin expression could serve as a marker for these "stem" cells. In addition, primary mammary cell cultures are



being evaluated for the efficiency of retroviral gene transfer, the level of recombinant *trans*-gene expression in the infected cultures, and the stability of recombinant retroviral gene expression in "mammary cell populations" subsequent to repopulation of the gland-free mammary fat pads. We have developed a number of partially transformed preneoplastic mammary outgrowth lines which are carried serially *in vivo* by transplantation into gland-free mammary fat pads. Cells from these lines have been grown *in vitro* and are capable of repopulating mammary fat pads subsequently. These cells are being tested for retroviral gene transfer studies.

#### The Identification and Characterization of Human Genes Associated with Neoplasia.

The etiology of human breast cancer is thought to involve a complex interplay of genetic, hormonal, and dietary factors that are superimposed on the physiological status of the host. Attempts to derive a cohesive picture of how these factors participate in the etiology of breast cancer have been confounded by a lack of information on specific mutations associated with the initiation and progression of the disease. We have undertaken a strategy that is aimed at determining, on a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant association of patient history, tumor characteristics, and patient postsurgical prognosis. The *int-2* gene is frequently activated in mouse mammary tumors by the mouse mammary tumor virus (MMTV) via insertional mutagenesis. We previously found that *int-2* is amplified 2- to 15-fold in 14% of primary human breast tumor DNAs (n = 118) and has a significant association ( $p < 2 \times 10^{-6}$ ) with a poor prognosis for the patient. We have now defined the amplification unit on chromosome 11q13 and found that 17 of 18 tumors amplified for *int-2* were also amplified for *hst* and *bcl-1*. The *hst* and *int-2* genes are both related to basic fibroblast growth factor. The *bcl-1* locus defines the translocation t(11;14) breakpoint in B-cell chronic lymphocytic leukemias. In a lymph node metastasis of a breast cancer patient, *int-2* but not *hst* was expressed. In 10% of the primary breast tumor DNAs (n = 122) the *c-erbB-2* gene was amplified. The *c-erbB-2* gene is related to the gene encoding the epidermal growth factor receptor. However, in contrast to one published report, we found no significant association with patient postsurgical prognosis or other aspects of patient history and tumor characteristics. In other studies, we detected the expression of MMTV-related human RNA sequences in breast tumor-derived cell lines and have obtained recombinant cDNA clones of these sequences for further analysis.

Cloning of Anti-Tumor Antigen Immunoglobulin Genes (The Summary of these studies appears under the Experimental Oncology Section [below] for continuity.)

#### CELLULAR AND MOLECULAR PHYSIOLOGY SECTION (H. Cooper, Chief)

Cytoskeletal Proteins in Oncogenic Transformation and Human Neoplasia. We are examining the relationship between neoplastic transformation and biochemical derangements of expression or utilization of tropomyosins (TMs). In NIH/3T3 fibroblasts transformed by a number of related and unrelated retroviral oncogenes, expression of the transformed phenotype was reproducibly correlated with suppression of synthesis of two specific TM isoforms ("muscle type"). Of all the proteins detectable on two-dimensional gel electrophoresis of whole-cell proteins, only the TMs behaved in this way. Treatment of rat fibroblasts with tumor growth factor- $\alpha$  (TGF- $\alpha$ ), in addition to inducing the transformed phenotype, specifically suppressed synthesis of muscle-type TMs and inhibited their utilization in the cytoskeleton, strengthening the connection between suppression of TM and expression of the transformed phenotype and adding support at the biochemical level for an autocrine pathway in oncogene action. The findings point out the close biochemical relationship between epidermal growth factor (EGF) receptor activation and microfilament organization. Tropomyosin expression

in relation to oncogene expression and neoplasia is being studied in murine and human epithelial cell systems. In mouse mammary epithelial cells transformed by the Ha-ras oncogene, the ratio of TM to actin accumulating in the cytoskeleton was reduced, indicating the production of microfilaments deficient in TM. The TM isoforms expressed by normal diploid human mammary epithelial cells have been compared with the pattern expressed by breast carcinoma cell lines. Defects in expression of particular TM isoforms occurred in all of the breast carcinoma cell lines studied, suggesting a possible role of deranged TM expression in human neoplasia.

Studies on the Nature and Function of the Phosphoprotein, Prosolin. We have extensively characterized prosolin, a major cytosolic protein of proliferating HL-60 promyelocytic leukemia cells that undergoes very rapid phosphorylation in response to treatment of the cells with tumor-promoting phorbol esters like TPA as an early step in a pathway leading to cessation of cell growth and onset of monocytoid differentiation. Prosolin has now been identified in proliferating human peripheral blood lymphocytes (PBL) by tryptic peptide mapping of proteins isolated from two-dimensional electrophoretic gels. While virtually absent from resting PBL, prosolin expression is induced by mitogens as a late event, during the onset of S-phase. Its level of synthesis correlates with the prevalent level of DNA synthesis. Prosolin undergoes rapid phosphorylation in proliferating PBL in response to TPA treatment, together with an inhibition of DNA synthesis. Phosphorylation of prosolin may be part of the mechanism by which naturally occurring growth inhibitory factors terminate normal lymphocyte proliferation. T-cell leukemia cell lines show a marked reduction in the rapidity of the phosphorylation of prosolin in response to TPA and other agents. The degree of reduction of the TPA-induced phosphorylation of prosolin in T-cell leukemias is related to the degree of undifferentiation of the neoplastic cells. We are cloning and sequencing cDNAs to prosolin. Partial amino acid sequence data were obtained by analysis of tryptic peptides of prosolin isolated from two-dimensional electrophoretic gels. Degenerate oligonucleotide probes were synthesized and used to probe a lymphoblast cDNA library. Positive clones are currently being analyzed.

#### ENDOCRINOLOGY WORKING GROUP (B. Vonderhaar)

Hormones and Growth Factors in Development of Mammary Glands and Tumorigenesis. The mammary gland is a complex organ whose growth and development is controlled by the interaction of a wide variety of hormones and growth factors. These same factors play fundamental roles in the etiology and progression of the cancerous state. The first event in the action of these hormones and growth factors is the interaction with specific cell associated receptors. The availability and activity of each class of receptor is regulated by the ligand which it recognizes as well as the general hormonal/growth factor milieu of the target cell. Our emphasis has been on the interactions of prolactin, thyroid hormone, and estrogens with recent work also examining how epidermal growth factor (EGF), and EGF-like growth factors are affected by the interplay of these three classical hormones. In addition we have explored the relationship of membrane associated antiestrogen binding sites to the lactogenic hormone receptor and the action of platelet derived growth factor (PDGF) on human breast cancer cell growth in culture. T47D cells have specific PDGF receptors and respond to its growth promoting signal by inducing phosphorylation of a 65Kd protein in the calelectrin family. Lobulo-alveolar development of the mammary gland requires the priming action of both estrogen and progesterone to induce EGF receptors and production of EGF-like growth factors. In concert with insulin, prolactin and glucocorticoids, EGF or  $\alpha$ -TGF can promote full lobulo-alveolar development *in vitro*. The primed mammary gland is more sensitive to  $\alpha$ -TGF than to EGF. Growth promotion of MCF-7 human breast cancer cells by prolactin can occur in the complete absence of estrogens. Prolactin induced growth of the estrogen receptor

negative Nb2 rat lymphoma cell can be blocked by non-steroidal antiestrogens such as tamoxifen. This action is through the membranous antiestrogen binding site (AEBS) which may be intimately associated with the prolactin receptor. The antiprolactin action of tamoxifen, working through the AEBS, may have important clinical implications.

#### EXPERIMENTAL ONCOLOGY SECTION (J. Schlom, Chief)

The studies being conducted within the Experimental Oncology Section can be divided into 8 major areas as detailed below. It should be emphasized that there is a great deal of cooperation and interaction among senior scientists involved in many of these projects.

Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens. These studies involve the generation, characterization, and utilization of monoclonal antibodies (MABs) directed against antigens associated with human carcinoma. These MABs are being used to better understand the cell biology and pathogenesis of several human carcinomas and to provide reagents that may be useful in several areas of the management of human carcinoma. These include *in vitro* diagnosis via serum assays and/or immunohistopathology, *in vivo* diagnosis such as gamma scanning, and potentially therapy. The MABs generated thus far can be classified into three groups based on the expression of the detected antigens. These are (a) antigens differentially expressed in human carcinoma versus normal adult tissues, such as the pancarcinoma tumor-associated glycoprotein (TAG)-72 which is detected by MAB B72.3, and carcinoembryonic antigen (CEA) which is detected by MABs COL-1 through COL-15; (b) tissue-associated antigens, such as the breast-associated antigen detected by MAB DF3, and the colon-associated antigens detected by MABs D612 and CAA; and (c) oncogene- or retroviral-related gene products, such as *int-2*.

Since MAB B72.3 has been shown to selectively target a range of carcinomas in clinical trials involving several hundred patients, studies were undertaken to characterize a series of "second generation" MABs to the TAG-72 antigen. Initial studies indicate that some of these second generation CC MABs, such as CC83 and CC49, have a higher affinity constant for TAG-72 than B72.3, and may be better suited than B72.3 for some clinical applications. A serologic map of TAG-72 is currently being constructed with 19 TAG-72 MABs. Studies are also currently in progress in the analysis of the COL MAB series and MAB D612 to define which MABs are best suited for *in vivo* clinical applications. Major emphasis is being placed on the generation and analyses of recombinant/chimeric (rec/chi) MABs versus TAG-72 and CEA and a rec/chi form of MAB D612, and modified constructs of the above rec/chi immunoglobulins. These rec/chi constructs and modified forms may be better suited for diagnostic and therapeutic applications and may help to better define mechanisms of antibody-directed cell-mediated cytotoxicity.

Characterization of Human Tumor-Associated Antigens. Human adenocarcinoma cells secrete and express on their cell surfaces high-molecular-weight, mucin-like glycoproteins that bear epitopes that are highly restricted to carcinoma cells. Monoclonal antibody B72.3, which has been extensively characterized as to reactivity with a variety of carcinomas versus normal tissues, reacts with such a mucin-like glycoprotein. The B72.3-reactive antigen, designated TAG-72, was partially purified and used as immunogen to produce second generation anti-TAG-72 antibodies, designated CC antibodies. Based on competitive binding studies, a number of the epitopes recognized by the anti-TAG-72 antibodies appear to be structurally or spatially related, but none of 18 analyzed appears to be identical. One of the second generation antibodies was selected to develop a procedure to purify preparative amounts of TAG-72. TAG-72, extracted from xenografted human colon carcinoma cells, was subjected to antibody-affinity, size-exclusion, and ion-exchange chromatography. A double determinant radio-



immunoassay revealed a greater than 1000-fold purification with a greater than 10% yield. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of radiolabeled purified TAG-72 revealed a polydisperse, but apparently homogenous, high-molecular-weight glycoprotein. The density of purified TAG-72 was 1.45 g/ml, indicating a high degree of glycosylation. Amino acid analysis of the purified TAG-72 revealed a profile highly similar to that reported for other purified mucins. The results of double immunodiffusion assays indicate that each epitope recognized by the anti-TAG-72 antibodies is expressed in multiple copies on the TAG-72 molecule. The purified TAG-72 has been partially deglycosylated with trifluoromethane sulfonic acid in preparation for amino acid sequence analysis. Other studies are underway to determine the physical location, coexpression, and chemical makeup of the various tumor-specific epitopes borne on the purified TAG-72; to analyze and compare TAG-72 derived from other sources, including other human solid tumors, pleural effusions, ascites fluids, serum, etc., with that derived from LS174T xenografts; and to compare the spectrum of expression of the various tumor-specific epitopes on TAG-72 derived from various sources.

Augmentation of Tumor Antigen Expression. Previous studies have shown that treatment of human carcinoma cells with type I (i.e., IFN- $\alpha$ ) or type II (i.e., IFN- $\gamma$ ) interferons increased the level of expression of several distinct tumor antigens (i.e., 90-kD antigen, CEA and TAG-72), resulting in an enhanced level of binding of monoclonal antibodies (Mab) B6.2, COL-1, and B72.3, respectively. IFN- $\alpha$  treatment of mice bearing breast or colorectal xenografts enhanced the localization of  $^{125}\text{I}$ -B6.2-F(ab')<sub>2</sub> several fold. Cells were also isolated from pleural or peritoneal effusions of patients diagnosed with adenocarcinoma, malignant nonepithelial tumors, and benign diseases and analyzed for constitutive TAG-72 and the ability of IFN- $\alpha$ , IFN- $\beta$ <sub>ser</sub>, and IFN- $\gamma$  to increase Mab B72.3 binding. TAG-72 expression was confined to those effusions isolated from patients diagnosed with metastatic adenocarcinoma (i.e., 35 of 43 effusions). Treatment with either type of interferon resulted in an increase in B72.3 binding in 27 of the 35 (77.1%) TAG-72 positive samples. The results provide further evidence that the interferon-mediated increase in human tumor antigen expression may be useful in the detection and/or treatment of occult tumor lesions.

Isolation and Characterization of Genes Coding for Carcinoma-Associated Antigens. Recent studies have demonstrated that CEA belongs to a multigene family related to the immunoglobulin supergene family. These include CEA, normal cross-reacting antigen (NCA), biliary glycoprotein (BGP), and human pregnancy-specific beta 1-glycoprotein (SP1). A 550-base pair CEA probe derived from cloned complementary DNA was used to carry out Southern analysis of the DNA isolated from normal and colon tumor cell lines. At high stringency, the CEA probe detected seven *Bam*HI fragments in all DNAs analyzed. Results of the Southern analysis demonstrate no amplification or rearrangement of the CEA genes in tumor cells. Methylation-sensitive restriction endonucleases, *Hpa*II and *Hha*I, were used to compare the degrees of methylation of the CEA family of genes in normal and colon tumor cell lines. The results demonstrate that the CEA family of genes exists in a state of hypermethylation in normal cells. In contrast, the CEA gene(s) are relatively hypomethylated in the tumor cell lines, suggesting a correlation between the state of methylation and degree of expression of the CEA gene(s). Using a CEA cDNA probe, we have investigated the phenomenon of up-regulation of CEA expression in human tumor cell lines by recombinant human gamma-interferon (Hu-IFN- $\gamma$ ). An increase in Mab binding was shown to be accompanied by a 6- to 11-fold increase in the steady-state levels of three CEA transcripts with sizes of 4.2, 3.5, and 2.8 kb. Normal human fibroblasts had no detectable cytoplasmic CEA glycoprotein levels nor did they contain detectable levels of CEA mRNA, either before or after treatment with Hu-IFN- $\gamma$ . Our observations suggest that some common factors may also be involved in the regulation of the CEA and class II



histocompatibility genes. Studies are also in progress to identify and characterize the gene(s) encoding the TAG-72 antigen.

Antibody Directed Cellular Immunotherapy of Colon Cancer. This project is examining the role that monoclonal antibodies (MAb) against human colonic tumor antigens have in directing and augmenting the cytotoxic activity of NK/LAK cells and T cells. A MAb designated D612 (IgG2a) has been generated that reacts homogeneously with the cell surface of approximately 80% of primary or metastatic colorectal carcinomas. Normal tissue reactivity of D612 is confined mainly to the small and large intestine, while non-gastrointestinal normal tissues are routinely negative. D612 mediated antibody-dependent cellular cytotoxicity in conjunction with normal human peripheral blood mononuclear cells was found to be increased by effector cell activation with interleukin 2 (IL-2). For retargeting of T cells, heteroantibody conjugates between the anti-colon tumor MAb, B38.1, and anti-CD3 (OKT-3) were chemically prepared, and these were tested along with effectors obtained from PBMC stimulated with anti-CD3 and IL-2. The heteroantibody-mediated cellular cytotoxicity activity was similar on a per cell basis among effector populations prepared by stimulation with IL-2, anti-CD3, or a combination of these reagents. However, the total lytic activity of anti-CD3 + IL-2-stimulated cultures was much higher due to the greater expansion in cell number achieved by the combination treatment. These studies suggest that MAbs with restricted normal tissue reactivity and ability to direct the cellular cytotoxicity of a broader spectrum of killer lymphocytes activated by lymphokines and other agents might augment the efficacy of colon cancer immunotherapy.

Cloning of Anti-Tumor Antigen Immunoglobulin Genes (Part of Oncogenetics Section, LTIB). The main objective of this research project is to genetically engineer immunoglobulin genes to generate useful reagents for *in vivo* localization and therapy of human tumors. The usefulness of mouse MAbs for *in vivo* therapy and diagnosis of human tumors is limited because of their immunogenicity in patients. This problem can be reduced by replacing the constant region of the mouse antibody with the constant region of the human antibody, using recombinant DNA techniques. To that end, we have cloned the rearranged heavy and light variable regions as well as the cDNA copies of the heavy and light chain gene messages of the MAb B72.3. Chimeric heavy and light chain immunoglobulin genes have been constructed. They were inserted separately into appropriate expression vectors. Stable chimeric antibody-producing cell lines were developed by introducing these constructs into both fibroblast and myeloma cell lines. Chimeric antibody produced either from cDNA clones or clones of the rearranged genes retained specificity and binding properties of the parental antibody. Studies are underway to develop modified constructs of recombinant/chimeric B72.3 as well as other anti-tumor MAbs.

Immunoassays for Human Carcinoma Associated Antigens and Localization and Therapy Using Labeled Monoclonal Antibodies: Model Systems. Immunoassays for the detection of circulating tumor associated antigens and assays for the support of clinical trials have been developed. We are using these assays to examine sera and effusions from a variety of sources to study their utility to detect carcinoma. We have developed a second generation of MAbs against a tumor-associated antigen, TAG-72, and used them in the development of a more efficient assay for TAG-72. This assay, designated CA 72-4 (using MAbs B72.3 and CC49) was developed in collaboration with Centocor and used to examine a panel of sera from patients with adenocarcinomas as well as benign diseases. Only 0.5% of 744 sera primarily obtained from blood donors had TAG-72 levels >10 U/ml. Similarly only 2.2% of 134 samples from patients with benign gastrointestinal diseases had TAG-72 levels >10 U/ml. Approximately 32% of 303 patients with gastrointestinal malignancies had serum TAG-72 >10 U/ml; approximately 44% of the patients with advanced disease had elevated TAG-72 levels. Patients with adenocarcinomas from a variety of other sites also had levels of TAG-72 above the reference value.

The potential utility of the CA 72-4 assay to be used in combination with CEA was demonstrated in patients with gastric cancer.

A number of new chelate-conjugates have been developed enabling the binding of radiometals to proteins. These are currently being tested in human tumor xenograft models. These new constructs have been covalently linked to B72.3 IgG to determine which resulted in the best biodistribution after labeling with In-111 and Y-88 (used as a substitute for Y-90). The 1-(p-isothiocyanatobenzyl) diethylenetriaminepentaacetic acid (SCN-Bz-DTPA) chelate-conjugate, gave the best biodistribution after labeling with either In-111 or Y-88. The biodistribution of the Y-88 and In-111 labeled MABs differed significantly, illustrating the difficulties in using In-111-labeled MABs to predict the distribution and dosimetry of Y-90-labeled MABs.

New anti-TAG-72 MABs (designated CC) have been developed and compared to B72.3 in the LS-174T model system. All exhibited higher %ID/gm and tumor:tissue ratios than B72.3; differences in the pharmacokinetics were noted among the CC MABs.

A recombinant/chimeric form of B72.3 has been developed using the variable regions of the murine B72.3 and human heavy chain ( $\gamma$ 4) and light chain ( $\kappa$ ) constant regions. The cB72.3( $\gamma$ 4) IgG was radiolabeled and the biodistribution was studied in athymic mice bearing LS-174T xenografts. Minor differences were observed between the cB72.3( $\gamma$ 4) and the native B72.3 in the %ID/gm that localized in the tumor.

Anti-Carcinoma Monoclonal Antibodies Clinical Trials. This project involves the use and the proposed use of several monoclonal antibodies (MABs) in both diagnostic and therapeutic collaborative clinical trials. To date, approximately 500 patients have been administered radiolabeled B72.3 in tumor-localization studies carried out in several institutions, with similar findings of approximately 70-80% tumor targeting observed.

We first investigated the administration of radiolabeled MAB B72.3 IgG in colorectal cancer patients. The selective localization of  $^{131}\text{I}$ -MAB B72.3 IgG was demonstrated in biodistribution studies in which the percentage of injected dose of MAB per gram of each tumor was compared with that of the normal tissues, thus providing a relative radiolocalization index (RI) for each lesion. Of the tumor lesions, 70% (99/142) had an RI of at least 3 (i.e., 3 times greater uptake per gram than normal tissues). We have also conducted studies to determine the feasibility of intraperitoneal administration of radiolabeled B72.3 for tumor localization (via both gamma scanning and direct analyses of biopsy specimens). Several patients studied were negative for tumor detection by both CT scan and X-ray but were positive for tumor localization via gamma scanning. Direct analyses of biopsy specimens of carcinoma and normal tissues demonstrated ratios of 3:1 to up to 70:1 for tumor MAB localization versus normal tissues.

A phase I therapy trial involving intraperitoneal administration of  $^{131}\text{I}$ -B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity has begun. The use of recombinant/chimeric MABs, second generation MABs, and new MAB-isotope conjugates are planned.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09018-05 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

## Anti-Carcinoma Monoclonal Antibodies Clinical Trials

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

Jeffrey Schlom	Chief	LTIB, DCBD, NCI
David Colcher	Supr. Microbiologist	LTIB, DCBD, NCI
Mario Roselli	Visiting Fellow	LTIB, DCBD, NCI
Jean Simpson	Medical Staff Fellow	LTIB, DCBD, NCI
Saed Mirzadea	Expert	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. J. Carrasquillo, CC, NM; Drs. A. Raubitschek and O. Gansow, ROB, DCT, NCI; Dr. E. Reed, COP, DCT, NCI; Dr. M. Merino, LP, DCBD, NCI; Dr. M. Lotze, SB, DCT, NCI; Dr. E. Martin, Ohio State Univ., Dept. Surg.; See attached sheet.

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Experimental Oncology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.6

## PROFESSIONAL:

2.1

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

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

A

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

This project involves the use and the proposed use of several monoclonal antibodies (MABs) in both diagnostic and therapeutic clinical trials. Thus far, all our collaborative clinical trials have been conducted with MAB B72.3. This MAB has been previously shown to have differential reactivity for a range of human carcinomas as compared to most normal adult tissues. To date, approximately 500 patients have been administered radiolabeled B72.3 in tumor-targeting studies carried out in several institutions, with similar findings of approximately 70-80% tumor targeting observed.

We first investigated the administration of radiolabeled MAB B72.3 IgG in colorectal cancer patients. The selective localization of <sup>131</sup>I-MAB B72.3 IgG was demonstrated in biodistribution studies in which the percentage of injected dose of MAB per gram of each tumor was compared with that of the normal tissues, thus providing a relative radiolocalization index (RI) for each lesion. Of the tumor lesions, 70% (99/142) had an RI of at least 3 (i.e., 3 times greater uptake per gram than normal tissues). We have also conducted studies to determine the feasibility of intraperitoneal administration of radiolabeled B72.3 for tumor localization (via both gamma scanning and direct analyses of biopsy specimens). Several patients studied were negative for tumor detection by both CT scan and X-ray but were positive for tumor localization via gamma scanning. Direct analyses of biopsy specimens of carcinoma and normal tissues demonstrated ratios of up to 70:1 for tumor MAB localization versus normal tissues.

A phase I therapy trial involving intraperitoneal administration of <sup>131</sup>I-B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity has begun. The use of recombinant/chimeric MABs and other MAB-isotope conjugates are planned.

Cooperating Units (continued):

Dr. M. Salvatore, NCI (Naples); Dr. D. Bigner, Duke Univ.; Dr. S. Larson,  
Memorial Sloan-Kettering, Nucl. Med.



### Major Findings

We have investigated the administration of radiolabeled MAb B72.3 IgG in colorectal cancer patients. These studies were possible due to a previously ongoing NCI Surgery Branch protocol for the treatment of patients with metastatic colorectal carcinoma, which involved the surgical resection of metastatic colorectal cancer lesions plus adjacent "normal" tissues for staging and therapeutic purposes. Thus, gamma scanning followed by a direct examination of tumor and a variety of normal tissues for MAb localization could be achieved. In our first trial, B72.3 IgG was labeled with <sup>131</sup>I and injected i.v. into 20 patients with known or suspected colorectal cancer. The selective localization of <sup>131</sup>I-MAB B72.3 IgG was demonstrated in biodistribution studies in which the percentage of injected dose of MAb per gram of each tumor was compared with that of the normal tissues, thus providing a relative radiolocalization index (RI) for each lesion. Of the tumor lesions, 70% (99/142) had an RI of at least 3 (i.e., 3 times greater uptake per gram than normal tissues), and 31% of the tumor lesions had RIs of over 10. Only 12 of 210 (6%) histologically normal tissues had RIs of >3; either these tissues were adjacent to or draining tumor masses or, as in the case of two patients, the high RI values were apparently due to deposition of immune complexes in spleen. Several parameters were studied to determine factors that might influence MAb localization. No differences in MAb uptake were observed among carcinoma lesions for numerous anatomic locations. No toxicity was observed. Additional studies employing <sup>111</sup>In-B72.3 coadministered with <sup>125</sup>I-B72.3 are in progress.

We have also conducted a series of collaborative studies with the Department of Surgery, Ohio State University (Dr. E. Martin) on the use of <sup>125</sup>I-radiolabeled B72.3 and an intraoperative hand-held probe, i.e., a gamma-detecting probe (GDP) to detect occult gastrointestinal, as well as other carcinoma lesions, at surgery. In initial studies using <sup>125</sup>I-B72.3 IgG, MAb was injected 5-42 days preoperatively, and the hand-held GDP was used intraoperatively to help detect the presence of tumor. Positive probe counts were detected in 5 of 6 (83%) patients with primary colon cancer, and in 31 of 39 (79%) patients with recurrent colon cancer.

We have conducted studies at the NIH to (a) determine the feasibility of intraperitoneal administration of radiolabeled B72.3 for tumor localization (via both gamma scanning and direct analyses of biopsy specimens); (b) determine the specificity of tumor localization by the concomitant i.p. administration of <sup>131</sup>I-labeled B72.3 and an isotope matched control <sup>125</sup>I-labeled MAB BL-3 (anti-idiotypic) IgG; (c) compare tumor localization of i.v. versus i.p. administered MAB by the simultaneous administration of <sup>125</sup>I-B72.3 IgG i.v. and <sup>131</sup>I-B72.3 IgG i.p.; and (d) define the pharmacokinetics of plasma clearance of both i.p. and i.v. administered radiolabeled MAB B72.3. At the time of entry into the protocol, all patients had confirmed or suspected metastatic colorectal peritoneal lesions. Subsequent studies have also been conducted with ovarian carcinoma patients with similar results. Four to seven days before surgery, all patients were administered <sup>131</sup>I-labeled B72.3 IgG i.p. via a catheter. Three of 10 patients studied were negative for tumor detection by both CT scan and X-ray but were positive for tumor localization via gamma scanning. Direct analyses of biopsy specimens of carcinoma and normal tissues demonstrated ratios of up to 70:1 (based on percentage of injected dose per kilogram) for tumor MAB localization versus normal tissues. Specificity of <sup>131</sup>I-B72.3 tumor targeting was demonstrated by the concomitant administration of <sup>125</sup>I-labeled isotype identical (IgG<sub>1</sub>) control MAB. Simultaneous i.p. administration of <sup>131</sup>I-B72.3, and i.v. administration of <sup>125</sup>I-B72.3 in individual patients demonstrated:

(a) peritoneal implants are targeted more efficiently via i.p. MAb administration, and (b) hematogenously spread and lymph node metastases as well as local recurrences are targeted more efficiently by i.v. administered MAb. No antibody toxicity was observed in any patients. Pharmacokinetics of MAb clearance demonstrated that only 10-30% of the i.p. administered MAb was found in plasma. These studies thus demonstrated the efficacy of intracavitary MAb administration as well as the advantage of the concomitant use of intracavitary and i.v. administered MAbs for tumor targeting and for potential MAb-guided therapy of metastatic carcinoma.

A phase I trial involving i.p. administration of  $^{131}\text{I}$ -B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity has begun at the NIH. These studies were initiated following dosimetry calculations that were based on analyses of pharmacokinetics and tumor and normal tissues sampling of diagnostic doses of i.p. administered  $^{131}\text{I}$ -B72.3.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05190-09 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens

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

Jeffrey Schlom	Chief	LTIB, DCBD, NCI
Patricia Horan-Hand	Chemist	LTIB, DCBD, NCI
Jean Simpson	Medical Staff Fellow	LTIB, DCBD, NCI
Alfredo Molinolo	Visiting Fellow	LTIB, DCBD, NCI
David Golcher	Microbiologist	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

D. Kufe, Dana Farber Cancer Center; P. Natali, Istituto Regina Elena, Rome;  
 M. Viola, SUNY at Stony Brook; W. Johnston and C. Szpak, Department of Pathology,  
 Duke University; M. Bodmer, Celltech, England

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Experimental Oncology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.6

## PROFESSIONAL:

1.6

## 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.)

These studies involve the generation, characterization, and utilization of monoclonal antibodies (MABs) directed against antigens associated with human carcinoma. These MABs are being used to better understand the cell biology and pathogenesis of several human carcinomas and to provide reagents that may be useful in several areas of the management of human carcinoma. These include *in vitro* diagnosis via serum assays and/or immunohistopathology, *in vivo* diagnosis such as gamma scanning, and potentially therapy. The MABs generated thus far can be classified into three groups based on the expression of the detected antigens. These are (a) antigens differentially expressed in human carcinoma versus normal adult tissues, such as the pancarcinoma tumor-associated glycoprotein (TAG)-72 which is detected by MAB B72.3, and carcinoembryonic antigen (CEA) which is detected by MABs COL-1 through COL-15; (b) tissue-associated antigens, such as the breast-associated antigen detected by MAB DF3, and the colon-associated antigens detected by MABs D612 and CAA; and (c) oncogene- or retroviral-related gene products, such as *int-2*.

Since MAB B72.3 has been shown to selectively target a range of carcinomas in clinical trials involving several hundred patients, studies were undertaken to characterize a series of "second generation" MABs to the TAG-72 antigen. Initial studies indicate that some of these second generation CC MABs, such as CC83 and CC49, have a higher affinity constant for TAG-72 than B72.3, and may be better suited than B72.3 for some clinical applications. A serologic map of TAG-72 is currently being constructed with 19 anti-TAG-72 MABs. Studies are also currently in progress in the analysis of the COL MAB series and MAB D612 to define which MABs are best suited for *in vivo* clinical applications. Major emphasis is being placed on the generation and analysis of recombinant/chimeric (rec/chi) MABs to both anti-TAG-72 and anti-CEA MABs and modified constructs of these rec/chi immunoglobulins. These rec/chi constructs and modified forms may be better suited for diagnostic and therapeutic applications.

### Major Findings

In view of the potential clinical applications of monoclonal antibody (Mab) B72.3 and our desire to further characterize the tumor-associated glycoprotein (TAG)-72 antigen, the decision was made to prepare "second generation" anti-TAG-72 Mabs. Because the source of purified TAG-72 used as immunogen was from a human colon cancer (CC) xenograft, Mabs were given a CC designation. Mice were immunized with purified TAG-72, and the resultant 2567 fusion cultures were assayed using solid-phase radioimmunoassays (RIAs) for reactivity to breast and colon cancer biopsies and for lack of reactivity to extracts of the following normal tissues: kidney, bone marrow, lung, liver, colon, stomach, thyroid, polymorphonuclear leukocytes, and erythrocytes. Ninety-nine cultures had the desired properties; these were cloned twice and assayed further. This process resulted in the selection of 28 hybridomas given a CC designation. These 28 CC Mabs were further tested in RIAs and shown to fall into several groups on the basis of differential reactivities to several carcinomas. The CC Mabs were also shown to be nonreactive to carcinoembryonic antigen (CEA) and to biopsy extracts of 32 normal human tissues. All 28 CC Mabs were tested using immunohistochemical analyses and shown to be reactive with formalin-fixed tissues (actually a consequence of a previous selection procedure). The 28 CC Mabs were positive to carcinoma sections and negative to the vast majority of cell types of a wide variety of normal tissues. In general, all the CC Mabs had patterns of reactivity for normal tissues very similar or identical to that shown and previously described for B72.3. The normal human adult tissue that showed strong staining with the CC Mabs (CC49 and CC83) and with B72.3 was secretory endometrium; proliferating endometrium and resting endometrium of postmenopausal women, however, were negative.

Nine CC Mabs were chosen for further study. These were CC11, 15, 29, 30, 40, 46, 49, 83, and 92. These Mabs were analyzed by Western blotting for reactivity to purified TAG-72 and showed the same pattern of reactivity as B72.3, recognizing a high-molecular-weight species of  $M_r > 10^6$ . The nine CC Mabs and B72.3 were analyzed as purified IgG preparations in several liquid competition RIAs to better define distinctions between binding sites of these Mabs. Mabs CC11, 15, 29, 30, 40, 46, 49, 83, and 92 could all be distinguished from one another. The affinity constants of the purified CC IgGs for binding to the TAG-72 antigen were determined; all CC Mabs chosen for further study had higher affinity constants than B72.3.

Studies were conducted to further characterize the range of reactivities of the anti-CEA COL Mabs. Fifteen competition RIAs, including the 14 COL-Mabs and 1 MAb reactive with NCA (a CEA related antigen), were developed and 15 purified competitor Igs were employed in each assay. Consequently, the 14 COL-Mabs were able to be categorized into six groups according to cross-competition patterns. The chemical nature of the epitopes identified by the COL-Mabs was also tested in solid-phase RIAs or Western blotting using chemically or enzymatically treated CEA. The results demonstrated that the epitopes identified by several of the COL-Mabs are not identical to each other. In an attempt to define which of the COL-Mabs are best suited for clinical applications, titration experiments were performed to define differential reactivities to colorectal carcinoma versus NCA-containing granulocyte extracts; these results were compared with results obtained using several anti-CEA Mabs that had been previously employed in clinical trials. Mabs COL-1, COL-4, COL-6, and COL-8 demonstrated a greater than  $10^5$ -fold differential for binding to a colon cancer versus granulocytes. These serological mapping and biochemical studies provide information as to the range and nature of the epitopes on the CEA molecule recognized by the COL-Mabs and provide additional evidence to form the basis for selecting the anti-CEA Mabs for

use in potential clinical applications, either alone or in combination with other anti-CEA MABs and MABs to other carcinoma-associated antigens.

We have recently generated and characterized a MAB, D612, with selective reactivity for malignant and normal gastrointestinal epithelium. MAB D612, a murine IgG<sub>2a</sub>, was generated using a membrane-enriched fraction of a human colon carcinoma biopsy as immunogen. Employing RIAs of biopsy extracts to a range of normal and neoplastic tissues and both immunofluorescence and immunoperoxidase assays on frozen sections of a range of normal and neoplastic tissues, we have shown that MAB D612 binds to 82% of the colorectal carcinomas tested ( $n = 67$ ) and to normal gastrointestinal epithelium but does not bind similarly to either neoplastic or normal tissues from a wide range of other sites. Western blotting has shown MAB D612 to react with a high-molecular-weight antigen. Live cell RIAs and fluorescence-activated cell sorter analyses demonstrated that the reactive epitope is present on the surface of 100% of the colon carcinoma cells tested. Immunohistochemical studies have shown intense membrane staining of colon adenocarcinomas; the vast majority of both primary and metastatic colon adenocarcinomas from a variety of sites were positive, with many lesions showing homogeneous staining of virtually all cells present. Using human effector cells, MAB D612 has been shown to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) of human colon carcinoma cells. Moreover, this activity was enhanced in the presence of interleukin (IL)-2. Radiolabeled D612-purified IgG was also shown to target a human colon carcinoma xenograft *in situ*. The pattern of membrane-associated staining, the molecular weight of the reactive antigen, the IgG<sub>2a</sub> isotype, the ability to mediate ADCC in the presence of IL-2, and the immunohistochemical and RIA studies demonstrating highly restricted reactivity to malignant and normal gastrointestinal tissue distinguish MAB D612 from other MABs thus far described.

One of our major efforts during the next few years will focus on the construction, expression, characterization, purification, and utilization of recombinant/chimeric (rec/chi) MABs. Initial focus will be on various constructs of B72.3, but these will be extended to the anti-TAG-72 MABs CC49 and CC83, the anti-CEA MAB COL-1, and MAB D612. These studies involve (a) methods of optimizing stability of cultures and Ig expression, (b) assays of the various rec/chi constructs, (c) *in vitro* binding properties of the various constructs, and (d) analyses of the rec/chi MABs for experimental *in vivo* tumor targeting and potential clinical trials. We have recently generated and characterized a rec/chi B72.3 of the human IgG<sub>4</sub> subtype. We have characterized this MAB with respect to binding properties to a range of normal human tissues and tumor tissues, and these studies indicate that the rec, rec/chi, and native B72.3 have virtually indistinguishable binding properties. We have also used an anti-idiotypic MAB prepared against B72.3 to show identical binding to native and rec/chi B72.3. Studies are in progress to prepare modified rec/chi constructs that may be better suited than native B72.3 for clinical applications.



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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09009-08LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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)

Jack Greiner	Cancer Expert	LTIB, DCBD, NCI
Fiorella Guadagni	Visiting Fellow	LTIB, DCBD, NCI
Shinya Shimada	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. S. Pestka, UMDNJ - Robert Wood Johnson Medical School, Piscataway, NJ;  
 Drs. E. Borden, D. Smalley, D. Goldstein, U. Wisconsin Med. Center, Madison WI;  
 Drs. W.W. Johnston, C. Szpak, Dept. of Pathology, Duke University, Durham, NC

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Experimental Oncology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.2

## PROFESSIONAL:

3.1

## OTHER:

1.1

## CHECK APPROPRIATE BOXES)

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

B

## SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

Previous studies have shown that treatment of human carcinoma cell with type I (ie., IFN- $\alpha$ ) or type II (ie., IFN- $\gamma$ ) interferons increased the level of expression of several distinct tumor antigens (ie., 90kD antigen, CEA and TAG-72), resulting in an enhanced level of binding of Mab B6.2, COL-1 and B72.3. IFN- $\alpha$  treatment of mice bearing breast or colorectal xenografts enhanced the localization of  $^{125}$ I-B6.2-F(ab'2) by 2-5 fold. Human tumor cells (ie., LS174T and A375) that were unresponsive in vitro showed no change in Mab localization in vivo as a result of IFN- $\alpha$  treatment. Higher and more stable circulating plasma interferon levels were achieved by using a constant flow pump and resulted in: 1) a 4-7 fold increase  $^{125}$ I-B6.2-F(ab'2) localization, 2) demonstration of that increase in Mab localization was time dependent and 3) a correlation between plasma IFN- $\alpha$  levels and the level of Mab localized. The ability of human interferons to increase the expression of CEA was also studied at the mRNA level using a cDNA probe. IFN- $\gamma$  treatment of moderately differentiated human colorectal tumor cells increased the binding of the anti-CEA Mab COL-4 2.5 and 6.5-fold which also was accompanied by a 6 to 11-fold increase in the steady state levels of three CEA related mRNA transcripts with sizes of 4.2, 3.5 and 2.8-kb. The results indicate that IFN- $\gamma$  regulation of CEA occurs at the transcriptional and/or posttranscriptional levels contributing to the enhanced level of expression of the antigen. Cells were also isolated from pleural or peritoneal effusions of patients diagnosed with adenocarcinoma, malignant nonepithelial tumor and benign diseases and analyzed for constitutive TAG-72 and the ability of IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$  to increase Mab B72.3 binding. TAG-72 expression was confined to those effusions isolated from patients diagnosed with metastatic adenocarcinoma (ie., 35 of 43 effusions). Treatment with either type of interferon resulted in a 50% increase in B72.3 binding in 27 of the 35 (77.1%) TAG-72 positive samples. The results provide further evidence that the interferon-mediated increase in human tumor antigen expression may be an effective adjuvant in the localization of Mab to occult tumor lesions.

Major Findings

We have reported that type I (i.e., IFN- $\alpha$ ) and type II (i.e., IFN- $\gamma$ ) human interferons are potent regulators of tumor antigens expressed on the surface of human breast and colorectal carcinoma cells. Initial studies revealed that in vitro treatment with IFN- $\alpha$ , or IFN- $\gamma$  resulted in a 3-6 fold increase in a 90kD tumor antigen, TAG-72 and CEA as measured by the binding of MAb B6.2, B72.3 and COL-1, respectively. Subsequent investigations revealed that the in vivo localization of radiolabeled B6.2 to a human colorectal xenograft in athymic mice could be increased by the administration of IFN- $\alpha$ . More recent studies investigated: 1) the parameters involved in the IFN- $\alpha$  mediated increase in MAB localization in athymic mice bearing human carcinoma xenografts, 2) the ability of IFN- $\gamma$  to increase the level of CEA-related mRNA transcripts in human colorectal tumor cells and 3) the regulation of TAG-72 and CEA expression by type I and type II interferons on human carcinoma and noncarcinoma cells isolated from patients' pleural and peritoneal effusions.

The initial study was designed to determine: 1) whether in vitro responsiveness of established human carcinoma cell lines was predictive of the ability of IFN- $\gamma$  to enhance in vivo MAB localization to that cell type grown as xenografts in athymic mice, 2) the advantage of delivering interferon in vivo via a constant flow osmotic pump and 3) whether any correlation exists between the circulating plasma IFN- $\alpha$  levels and the amount of radiolabeled MAB localized to the tumor site. WiDr, LS174T, A375 and the transplantable human mammary Clouser tumors were inoculated s.c. into athymic mice. The administration of IFN- $\alpha$  is twice daily i.m. injections resulted in the increase in  $^{125}\text{I-B6.2-F(ab')}_2$  localization to the Clouser and WiDr tumors. For example, athymic mice bearing the human mammary Clouser tumor were given daily i.m. injections of 250,000 antiviral units of IFN- $\alpha$  for 7 days. The mean percent injected dose of  $^{125}\text{I-B6.2}$  localized to the Clouser tumor rose from 6.9% to 12.8% as a result of IFN- $\alpha$  treatment. The range for the percent injected dose of  $^{125}\text{I-B6.2-F(ab')}_2$  for the Clouser bearing untreated mice was 3.9% to 8.0%; that for the IFN- $\alpha$  treated mice was 3.9% to >18%. In contrast, the human tumor cell lines (i.e., LS174T and A375) previously shown either to be unresponsive to the ability of IFN- $\alpha$  to enhance antigen expression or tumor antigen negative in vitro remained so in the in vivo studies. These results suggest that an in vivo screening analyses of human tumor cell populations may be possible for selection of patients for subsequent antigen augmentation therapy.

Pharmacokinetic studies revealed that i.m. administration of IFN- $\alpha$  resulted in a plasma half-life of 3-4 hrs. It was postulated that a more sustained plasma IFN- $\alpha$  level might result in a more dramatic increase in radiolabeled MAB localized to the tumor site. IFN- $\alpha$  was loaded into constant flow mini-osmotic pumps and implanted into athymic mice bearing human xenografts. Mice were bled at different time intervals for 7 days. The constant flow osmotic pumps maintained IFN- $\alpha$  levels of 300-800 antiviral units/ml for 6-7 days. Furthermore, the pumps could be replaced permitting analysis of  $^{125}\text{I-MAB}$  localization to human xenografts in vivo over an extended time interval. Athymic mice bearing WiDr tumors were treated with IFN- $\alpha$  administered via the osmotic pump for 9 and 13 days, respectively. The increase in  $^{125}\text{I-B6.2-F(ab')}_2$  localized to the tumor rose with the length of time of IFN- $\alpha$  treatment. For example, after a 9 day treatment of IFN- $\alpha$ , the mean percent injected dose of  $^{125}\text{I-B6.2-F(ab')}_2$  per gram of tumor was increased >250% above that for the untreated mice (i.e., 3.1 vs 7.7%). A 13-day IFN- $\alpha$  treatment increased the percent injected dose per gram tumor of  $^{125}\text{I-B6.2-F(ab')}_2$  to 10.9%; 3.5-fold



increase above that for the untreated group. An analysis of individual mice treated for 13 days with IFN- $\alpha$ A revealed that in some >15% of the injected dose of the MAb was localized to the tumor site. The amount of radiolabeled B6.2-F(ab')<sub>2</sub> localized (i.e., cpm/tumor weight) to the WiDr tumors in athymic mice was compared with the circulating plasma levels in each animal. Although a considerable difference was evident among the various mice, there was a correlation between plasma IFN- $\alpha$ A levels and the amount of <sup>125</sup>I-B6.2-F(ab')<sub>2</sub> localized to the tumor site.

Until recently the studies of the regulation of tumor antigens by human interferons have relied on the analysis of antigen levels measured by the binding of MAbs. Several laboratories have now reported the nucleotide sequence of several cDNA clones for the family of CEA-related genes. In collaboration with Drs. J. Kantor and P. Robbins, we studied the effect of IFN- $\gamma$  on the steady state levels of CEA mRNA and the level of CEA expression as measured by the binding of MAb COL-4 in human colorectal tumor cells. HT-29, WiDr and LS174T express different amounts of CEA as measured by the binding of MAb COL-4 and other quantitative assays. Treatment of the moderately differentiated colorectal cells, HT-29 and WiDr, with 1,000-2,000 antiviral units of IFN- $\gamma$ /ml for 48-72 hrs increased COL-4 binding by 2.5 and 6.5 fold, respectively. Using a 550-bp Pst I fragment CEA cDNA probe, the increase in COL-4 binding was accompanied by a 6- to 13-fold increase in the steady state levels of the three CEA-related transcripts of 4.2, 3.5 and 2.8 kb. In contrast, the highly differentiated colorectal human cell line, LS174T, showed no significant change in either COL-4 binding or the expression of the CEA-related transcripts as a result of IFN- $\gamma$  treatment. Normal diploid human fibroblasts, WI-38 and MRC-5, did not express constitutive CEA or the CEA-related mRNA transcripts either before or after IFN- $\gamma$  treatment. Previous studies have shown that IFN- $\gamma$  is a potent inducer of de novo class II HLA expression on a variety of human cells. Indeed, IFN- $\gamma$  treatment did induce the HT-29 and WiDr cells to express the class II HLA, however, the LS174T cells remained Class II HLA negative. The results indicate that IFN- $\gamma$  treatment results in an increase in the steady state levels of CEA mRNA indicating the regulation of that antigen at at transcriptional and/or post transcriptional levels. The IFN- $\gamma$  induced increase in CEA was accompanied by a concomitant increase in the steady state levels of the three transcripts. It is unclear whether the increase in CEA mRNA levels resulted from an increased rate of transcription of the CEA gene(s) and/or from the stabilization of the CEA message(s). Further studies are planned to address these and other issues concerning the biological relevance of the regulation of a human tumor antigen by IFN- $\gamma$ .

To date, the study of the regulation of TAG-72 by the recombinant human interferon has been confined to the human breast tumor cell line, MCF-7. TAG-72 is not well expressed on adherent human cell lines, and the other TAG-72 positive cell line, LS174T, is not responsive to the ability of type I or type II interferons to enhance tumor associated and normal (i.e., HLA) antigens (see above). Studies have shown that TAG-72 can be found in a high percentage of human tumor cells isolated from serous effusions of patients diagnosed with metastatic adenocarcinoma. A total of 61 serous effusions from patients diagnosed with adenocarcinoma (43), malignant non-epithelial tumors (10) and benign (8) diseases were analyzed for the expression of TAG-72, CEA and MHC antigen as well as the ability of type I and type II interferons to enhance their level(s) of expression. TAG-72 was found on approximately 80% (i.e., 35 of 43) of the



serous effusions isolated from patients with metastatic adenocarcinoma. None of the 10 effusions from patients with non-epithelial malignancies and 0 of 8 effusion from patients with other diseases (i.e., benign) were found to contain B72.3-reactive cells. Dose and temporal dependent increases in tumor-associated as well as class II HLA after IFN- $\gamma$  treatment agreed with previous results published for established cell lines. Of the 35 of 43 effusions from patients with metastatic adenocarcinoma that also contained TAG-72 positive tumor cells, treatment with either type I (i.e., IFN- $\alpha$ A or IFN- $\beta$ <sub>ser</sub>) or type II (i.e., IFN- $\gamma$ ) significantly increased B72.3 binding (i.e., 50% above that of untreated cells) in 77% of the cases (i.e., 27 of 35). Constitutive TAG-72 expression was not, however, a sine qua non requirement for its subsequent augmentation by human interferons. In at least 4 of 8 cases, the isolated malignant cells expressed high levels of TAG-72 and, a further increase in B72.3 binding could not be demonstrated after interferon treatment. We were also interested to determine whether treatment of those TAG-72 negative malignant cell populations might enhance the expression of another tumor antigen. In nine such cases, treatment of the malignant cell population with either IFN- $\alpha$ A or IFN- $\gamma$  resulted in a significant increase in CEA expression. Thus, 42 of 43 tumor cell populations isolated from patients with metastatic adenocarcinoma constitutively expressed either TAG-72 and/or CEA. Furthermore, in vitro incubations of the cells in the presence of type I or type II human interferons substantially increased the expression of one or both of the tumor antigens in 36 of the 42 cases (85.7%). The findings may provide important insight into the criteria for the selection of patients for antigen augmentation therapy: 1) cell surface tumor antigen expression and 2) ability of interferon treatment to augment MAb binding in vitro. In addition, these and previous finding provide important data to design clinical trials to evaluate the interferons as adjuvants in the localization of occult tumors with conjugated MAbs.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05233-08 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Immunoassays for Human Carcinoma Associated Antigens and Clinical Studies

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

David Colcher	Supr. Microbiologist	LTIB, DCBD, NCI
Patrizia Ferroni	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. A. Raubitschek, ROB, DCT, NCI; Drs. J. Carrasquillo, J. Reynolds, Div. Nucl. Med., NIH.; Drs. C. Szpak, W. Johnston, Dept. Path., Duke U. School of Med.; Dr. N. Ohuchi, Dept of Surg., Tohoku U. School of Med., Dr. P. Kaplan, Centocor, Inc.

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Experimental Oncology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

1.6

## OTHER:

0.9

## CHECK APPROPRIATE BOX(ES)

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

A

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

Immunoassays for the detection of circulating tumor associated antigens and assays for the support of clinical trials have been developed. We are using these assays to examine sera and effusions from a variety of sources to study their utility to detect carcinoma. We have developed a second generation of MAb's against a tumor-associated antigen, TAG-72, and used them in the development of a more efficient assay for TAG-72. This assay, designated CA 72-4 (using MAb's B72.3 and CC49) was developed in collaboration with Centocor and used to examine a panel of sera from patients with adenocarcinomas as well as benign diseases. Only 0.5% of 744 sera primarily obtained from blood donors had TAG-72 levels >10 U/ml. Similarly only 2.2% of 134 samples from patients with benign gastrointestinal diseases had TAG-72 levels >10 U/ml. Approximately 32% of 303 patients with gastrointestinal malignancies had serum TAG-72 levels >10 U/ml; approximately 44% of the patients with advanced disease had elevated TAG-72 levels. Patients with adenocarcinomas from a variety of other sites also had levels of TAG-72 above the reference value. The utility of the CA 72-4 assay was clearly demonstrated in the case of patients with gastric cancer where CEA levels were measured along with the TAG-72 levels. CEA was elevated in 37% of patients with Stage 3 and 4 disease and 48% of the sera had elevated levels of TAG-72. No relationship was observed between the positivity in the CEA assay and the CA 72-4 assay. Thus the use of both assays to analyze these sera is clearly more advantageous than using the CEA or the CA 72-4 assay alone.

The interaction in vivo of TAG-72 with radiolabeled MABs was also studied to aid in understanding the pharmacology of MABs used as radiopharmaceuticals. We found immune complex formation, even in the earliest sera drawn, as detected both by HPLC and SDS-PAGE analysis. Three different profiles, dependent on the serum TAG-72 level, were seen in the HPLC patterns. The presence of immune complexes did not appear to alter the clearance pattern of the I-131-MAB.

Major Findings

The development of immunoassays for the detection of circulating tumor associated antigens and the study of the interaction in vivo of these antigens with radio-labeled MAbs may help in the diagnosis of cancer, the monitoring of the clinical course of the disease, as well as understanding the pharmacology of the MAbs used as radiopharmaceuticals.

Mab B72.3, a murine IgG<sub>1</sub>, has been developed in this laboratory and shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, including colon, ovarian, breast, lung, pancreatic and gastric malignancies. We have purified the TAG-72 antigen and prepared a second generation of anti-TAG-72 MAbs. One of these MAbs, designated CC49, was shown to react with an epitope on TAG-72 that could be distinguished from that recognized by B72.3. Moreover, while the  $K_d$  of B72.3 was shown to be  $2.5 \times 10^9 M^{-1}$ , that of CC49 was considerably higher at  $16.2 \times 10^9 M^{-1}$ . These findings led to the development of a more efficient assay for TAG-72 using both MAb CC49 and B72.3; CC49 was used as the "catcher" and  $^{125}I$ -B72.3 as the detecting reagent. This combination enabled the development of a sequential assay that could be performed in less than one day which also minimized the "hook-back" effect and yielded the most reproducible recovery of TAG-72 in dilution studies. This is especially important in accurately quantitating TAG-72 levels in patient sera containing high levels of TAG-72.

The CA 72-4 assay was used to examine a panel of sera from patients with malignancies from a number of sites as well as benign diseases. Normal sera obtained primarily from blood donors was examined and only 4 of 744 samples (0.5%) tested had TAG-72 levels  $>10$  U/ml. Similarly, only 3 of 134 samples (2.2%) of patients with benign gastrointestinal diseases had TAG-72 levels  $>10$  U/ml. Whether the cut-off level of 10 U/ml is appropriate for use, or a different level such as 6 U/ml giving greater sensitivity or 20 U/ml giving greater specificity, will have to be further examined with much larger panels of sera from "normal individuals" and patients with benign diseases.

Approximately 40% of 303 patients with gastrointestinal malignancies had serum TAG-72 levels of  $>6$  U/ml (approximately 55% of patients with advanced disease). Whether the expression of TAG-72 in the sera of patients correlates with stage of disease needs to be further investigated with a well characterized set of sera. Approximately 36% of patients with lung adenocarcinomas and 24% of patients with ovarian cancer also had elevated serum TAG-72 levels; 53% of patients with Stage IV ovarian cancer had elevated TAG-72 levels. The utility of the CA 72-4 assay to measure TAG-72 levels was clearly demonstrated in the case of patients with gastric cancer. These sera were also evaluated in a RIA for CEA where 37% of patients with Stage 3 and 4 disease had elevated levels of CEA. The RIA for TAG-72 was positive in 48% of the sera tested from patients with gastric carcinomas. No relationship was observed between elevated TAA levels of sera in the CEA assay and the CA 72-4 assay. Thus, the use of both assays to analyze these sera is clearly an advantage to using the CEA or the CA 72-4 assay alone.

Radiolabeled B72.3, intravenously or intraperitoneally administered, has been shown to localize primary and metastatic lesions of colorectal and ovarian patients as well as patients with other carcinomas. Intravenous administration to colorectal carcinoma patients of  $^{131}I$ -B72.3 IgG has resulted in localization



of the MAb in approximately 75% of the metastatic lesions found at various body sites. We analyzed the serum of colorectal carcinoma patients who have received  $^{131}\text{I}$ -B72.3 IgG by intravenous administration. The presence of TAG-72, the pharmacokinetics of the radiolabeled MAB, the amount of circulating immunoreactive MAB B72.3 IgG, the status of the radionuclide and its association with the MAB were studied. The integrity of the radiolabeled B72.3 IgG in the serum and the formation of immune complexes were also analyzed. Plasma clearance of  $^{131}\text{I}$ -B72.3 IgG was determined for all patients and was similar in 25/27 patients; the mass of antibody injected had no effect on the rate of clearance of the radiolabeled B72.3 IgG. The level of immunoreactive B72.3 IgG in patient sera was determined using a solid-phase radioimmunoassay. When the level of immunoreactive antibody was normalized to the sera drawn 5 min post-injection, it was found that clearance of the immunoreactive antibody was similar to the clearance of the radiolabeled MAB B72.3. This suggests that the radionuclide is remaining in association with MAB B72.3 following administration to patients. This is further supported by HPLC analysis and autoradiography of the SDS-polyacrylamide gels run on the patients' sera which demonstrated that the radioactivity remains in association with the IgG fraction. HPLC analysis of the sera revealed three different profile patterns. The majority of the patients were in the category in which the radiolabel eluted with a retention time consistent with that of IgG. In others, a small percentage of the radioactivity eluted in the void volume, while in a third group, a large proportion of the radioactivity was associated with the void volume. Elution of the  $^{131}\text{I}$ -B72.3 in the void volume suggests that there is the formation of immune complexes or aggregation of the MAB. The formation of this higher molecular weight entity correlates with elevated antigen levels in patient sera, suggesting that antigen-antibody complexes are forming. We found that immune complex formation occurred even in the earliest sera drawn. Furthermore, immune complex levels remained consistent in the sera drawn at various intervals. These complexes were detected both by HPLC and SDS-PAGE analysis. Interestingly, the presence of the immune complexes did not appear to alter the clearance pattern of the monoclonal antibody. It was of interest also that serum TAG-72 levels correlated with obtaining a positive scan. Serum TAG-72 levels may be a useful criteria for considering a patient for treatment with MAB B72.3.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

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## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Localization and Therapy Using Labeled Monoclonal Antibodies: Model Systems

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

David Colcher	Supr. Microbiologist	LTIB, DCBD, NCI
Mario Roselli	Visiting Fellow	LTIB, DCBD, NCI
Patrizia Ferroni	Visiting Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Drs. O. Gansow and A. Raubitschek, ROB, DCT, NCI

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Experimental Oncology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.1

## OTHER:

0.1

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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.)

MAb B72.3, a murine IgG1, has been shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, with minimal reactivity to normal adult tissues. B72.3 was therefore chosen as an antibody that could potentially be used for radioimmunodetection (RID) and radioimmunotherapy (RIT) of human carcinomas and is being evaluated in model systems using the LS-174T human colon carcinoma xenograft to determine the feasibility of these studies. A number of new chelate-conjugates have been developed enabling the binding of radiometals to proteins. These new constructs have been covalently linked to B72.3 IgG to determine which resulted in the best biodistribution after labeling with In-111 and Y-88 (used as a substitute for Y-90). The 1-(p-isothiocyanatobenzyl)diethylenetriaminepentaacetic acid (SCN-Bz-DTPA) chelate-conjugate, gave the best biodistribution after labeling with either In-111 or Y-88. The biodistribution of the Y-88 and In-111 labeled MAb's differed significantly, illustrating the difficulties in using In-111-labeled MAb's to predict the distribution and dosimetry of Y-90-labeled MAb's.

New anti-TAG-72 MAb's (designated CC) have been developed and compared to B72.3 in the LS-174T model system. All exhibited higher %ID/gm and tumor:tissue ratios than B72.3; differences in the pharmacokinetics were noted among the CC MAb's.

A recombinant/chimeric form of B72.3 has been developed using the variable regions of the murine B72.3 and human heavy chain ( $\gamma$ 4) and light chain ( $\kappa$ ) constant regions. The cB72.3( $\gamma$ 4) IgG was radiolabeled and the biodistribution was studied in athymic mice bearing LS-174T xenografts. Small differences were observed between the cB72.3( $\gamma$ 4) and the native B72.3 in the %ID/gm that localized in the tumor, this is mostly likely due to its more rapid clearance from the blood and body of the mouse of the cB72.3( $\gamma$ 4) as compared to the native B72.3.



Major Findings

Monoclonal antibody (Mab) B72.3, a murine IgG<sub>1</sub>, has been developed in this laboratory and been shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, including colon, ovarian, breast, lung, pancreatic and gastric malignancies. <sup>131</sup>I-B72.3 IgG has been used successfully in vivo for the detection of human malignancies including colorectal, ovarian, breast and lung carcinomas after both i.v. and i.p. administration. B72.3 was therefore chosen as the antibody to use for the evaluation of different radiolabeling methodologies and MAb modification techniques that may alter the biodistribution of MAbs.

Recent interest has focused on the application of radiolabeled MAbs directed against tumor-associated antigens as in vivo therapeutic reagents. Labeling techniques have been developed that yield covalent linkage of chelates to proteins thus permitting the attachment to MAbs of many different radiometals that are considered to be useful for radioimmunotherapy. <sup>90</sup>Y has been selected as one of particular interest for therapeutic applications; it can be complexed with several chelate groups such as DTPA and EDTA which may be linked to proteins using a variety of methodologies. The fact that <sup>90</sup>Y is a pure  $\beta$ -emitter makes it difficult to estimate the radiation dose to a given tissue because the radioactivity accumulated in tissues cannot be accurately detected by external imaging nor can it be accurately quantitated using gamma or beta scintillation counters. To overcome this problem, the  $\gamma$ -emitting <sup>111</sup>In has been proposed as a tracer for yttrium in biodistribution studies. We have initiated studies to compare the biodistribution of yttrium and indium labeled Mab B72.3 using 3 different chelate-conjugates 1-(p-isothiocyanatobenzyl)diethylenetriaminepentaacetic acid (SCN-Bz-DTPA), 1-(p-isothiocyanatobenzyl)ethylenediaminetetraacetic acid (SCN-Bz-EDTA) and the cyclic anhydride derivative of DTPA (CA-DTPA) to determine if the <sup>111</sup>In can be used to accurately predict the <sup>90</sup>Y localization and therefore allow accurate dosimetric calculations. For these studies the  $\gamma$ -emitting yttrium radionuclide <sup>88</sup>Y was chosen in order to facilitate the biodistribution analysis employing a well-type gamma counter.

Athymic mice bearing LS-174T human colon carcinoma xenografts were injected via tail vein with Mab B72.3 IgG modified by the 3 different chelate-conjugates and labeled either with <sup>88</sup>Y or <sup>111</sup>In. The uptake in the tumor was highest with <sup>88</sup>Y-SCN-Bz-DTPA-MAB (40 %ID/gm at 5-7 days post-injection) compared to the 2 other chelate-conjugates, SCN-Bz-EDTA and CA-DTPA (only 6-8 %ID/gm). This is due in part to the rapid drop in the blood levels for the <sup>88</sup>Y-labeled SCN-Bz-EDTA and CA-DTPA conjugated MAbs in contrast to the <sup>88</sup>Y-SCN-Bz-DTPA-B72.3. The <sup>88</sup>Y-SCN-Bz-EDTA and <sup>88</sup>Y-CA-DTPA-B72.3 chelate-conjugate showed higher liver, spleen and kidney:blood ratios than the <sup>88</sup>Y-SCN-Bz-DTPA-B72.3. The differences in the tissue uptake of the 3 Mab chelate-conjugates is consistent with the release of the yttrium from the circulating CA-DTPA and SCN-Bz-EDTA Mab chelate-conjugates as evidenced by the high uptake in the bone, liver, spleen and kidney which is similar to the biodistribution seen after injection of yttrium acetate. Yttrium in its uncomplexed form tends to accumulate mainly in the bone resulting in bone marrow toxicity. Therefore, particular attention was to be paid to the evaluation of the yttrium levels in the bone. The amount of <sup>88</sup>Y in the bone rose as a function of time in mice injected with the SCN-Bz-EDTA and CA-DTPA-B72.3 resulting in over 14 and 11 %ID/gm at 5 days respectively. The levels of yttrium in the bones of these animals were comparable to those observed in animals given



$^{88}\text{Y}$ -acetate. In contrast, the mice injected with  $^{88}\text{Y}$ -SCN-Bz-DTPA-B72.3 showed significantly lower levels of  $^{88}\text{Y}$  in the bone (3 %ID/gm) indicating a better stability of this complex *in vivo*. The bone uptake for  $^{111}\text{In}$ -labeled B72.3 using all 3 chelate-conjugates was only 2-3 %ID/gm, similar to that found with the  $^{88}\text{Y}$ -SCN-Bz-DTPA-B72.3.

The differences between yttrium and indium biodistribution after administration of labeled MABs illustrates the difficulties in using  $^{111}\text{In}$ -labeled MABs to predict the biodistribution and dosimetry of  $^{90}\text{Y}$ -labeled MABs. Although a given chelate-conjugate may give a favorable biodistribution with  $^{111}\text{In}$ , the biodistribution of yttrium in that chelate-conjugate may be very different. These studies also demonstrated the importance of using the appropriate chelate for attaching yttrium and indium to the MAB. With the use of chelates such as SCN-Bz-DTPA, one can minimize loss of the radiometal from the chelate and obtain similar biodistributions using both indium and yttrium. The low level of bone uptake of the yttrium when the SCN-Bz-DTPA chelate-conjugate is used is especially important in minimizing marrow toxicity. The high tumor to bone ratios suggest the potential utility of  $^{90}\text{Y}$  for radioimmunotherapy using this type of chelate-conjugate.

One problem that has been encountered with the *in vivo* use of both the polyclonal and monoclonal antibodies is the patients' development of an immune response to the administered antibody. Greater than 50% of the patients that have been administered B72.3 have developed an immunological response to murine IgG after a single injection. The use of fragments of MABs has led to a decrease in the incidence of an anti-mouse Ig response in the patients. Most IgG fragments, when used *in vivo*, however, have shown a much lower %ID/gm in the tumor as compared to the intact IgG. Another way to minimize the patients' immune response to the injected antibody is to use human or recombinant/chimeric MABs. We have developed a recombinant form of the murine B72.3 as well as a recombinant/-chimeric antibody, using the variable regions of the murine B72.3 and human heavy chain ( $\gamma_4$ ) and light chain ( $\kappa$ ) constant regions. Both the recombinant B72.3 (rB72.3) and the recombinant/chimeric B72.3 (cB72.3( $\gamma_4$ )) IgGs maintain the tissue binding and idiotypic specificity of the native murine IgG. The native B72.3, rB72.3 and cB72.3( $\gamma_4$ ) IgGs were radiolabeled and the biodistribution of these IgGs was studied in athymic mice bearing LS-174T xenografts. Differences were observed between the cB72.3( $\gamma_4$ ) and the native B72.3 in the %ID/gm that localized in the tumor. The somewhat lower absolute amounts of the cB72.3( $\gamma_4$ ) in the tumor are mostly likely due to the more rapid clearance of the cB72.3( $\gamma_4$ ) from the blood and body of the mouse as compared to the native B72.3 and rB72.3. All three forms (native B72.3, rB72.3 and cB72.3( $\gamma_4$ )) of the IgG, however, were able to localize the LS-174T xenografts with similar radiolocalization indices.

The B72.3 reactive antigen, TAG-72, has been purified and a series of second generation MABs, designated CC (colon cancer), have been characterized by a range of *in vitro* immunological assays. Six CC MABs (CC11, CC30, CC46, CC49, CC83 and CC92) were chosen for analyses of the *in vivo* binding to the LS-174T human colon carcinoma xenografts. All 6 MABs were previously shown to be distinct from B72.3 and each other by a series of reciprocal competition RIAs, and all were shown to have higher  $K_a$ s than B72.3. All six CC MABs evaluated were superior to B72.3 in an *in vivo* tumor targeting model, in terms of both the percentage of the injected dose of radiolabeled MAB delivered per gram of tumor and in the radiolocalization indices (RIs). Differences in the *in vivo* binding patterns and pharmacokinetics among the CC MABs have also been evaluated.

The CC MABs demonstrated several different clearance patterns. One fairly unique pattern was displayed by MAB CC49. The plasma clearance of CC49 was much faster than that of B72.3 and the five other CC MABs analyzed. This resulted in much higher tumor:blood RIs for CC49 than for the other MABs. There were also differences in the RIs of the other normal tissues examined. For B72.3 and the other CC MABs the RIs can be ranked as follows: tumor:liver, spleen and kidney showed the highest RIs, then tumor:lung and lastly tumor:blood. For CC49, however, the order of the RIs (from high to low) were tumor:kidney, lung, blood, spleen and liver. One possible explanation for this phenomenon is that CC49 recognizes an epitope of TAG-72 which is highly accessible when TAG-72 is found in circulation, and the resulting MAb-TAG-72 complexes deposit in the liver and spleen, thus lowering the RIs for these organs. The MAb therefore clears the plasma rapidly. Further evidence for this is found in the observation that when CC49 is administered to mice with small tumors the ranking of the RI values (notably tumor:spleen and tumor:liver) of the tissues is similar to that observed for B72.3 and the other CC MABs. Regardless of this phenomenon and the resulting ranking of RIs for tumor to various normal organs, the rapid plasma clearance of the CC49 resulted in CC49 demonstrating among the highest RI values for the CC MABs. The potential clinical advantage of using the CC MABs for in vivo tumor targeting for either diagnostic or therapeutic applications is supported by both the higher %ID/gm of tumor and the higher RI values of the second generation CC MABs as compared to that of B72.3.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

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

Isolation and Characterization of Genes Coding for Carcinoma-Associated Antigens

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

Judy Kantor	Cancer Expert	LT1B, DCBD, NCI
Paul Robbins	Senior Staff Fellow	LT1B, DCBD, NCI
Scott Meissner	Staff Fellow	LT1B, DCBD, NCI
Chrysanthi Paranavitana	Fogarty Fellow	LT1B, DCBD, NCI
Howard Kaufman	Biotechnology Fellow	LT1B, DCBD, NCI

COOPERATING UNITS (if any)

S. Pestka, Robert Wood Johnson Medical School, Piscataway, NJ; J.E. Shively, Beckman Research Institute of the City of Hope, Duarte, CA; P.B. Fisher, Institute of Cancer Research, Columbia University, NY

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

5.1

PROFESSIONAL:

5.1

OTHER:

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) have defined several antigens associated with human carcinomas. Two of the most widely studied antigens are carcinoembryonic antigen (CEA) and TAG-72. CEA is a 180,000-kd glycoprotein and TAG-72 is a high-molecular-weight mucin. Recent studies have demonstrated that CEA belongs to a multigene family related to the immunoglobulin supergene family. These include CEA, normal cross-reacting antigen (NCA), biliary glycoprotein (BGP), and human pregnancy-specific beta 1-glycoprotein (SP1). A 550-base pair CEA probe derived from cloned complementary DNA was used to carry out Southern analysis of the DNA isolated from normal and colon tumor cell lines. At high stringency, the CEA probe detected seven *Bam*HI fragments in all DNAs analyzed. Results of the Southern analysis demonstrate no amplification or rearrangement of the CEA genes in tumor cells. Methylation-sensitive restriction endonucleases, *Hpa*II and *Hha*I, were used to compare the degrees of methylation of the CEA family of genes in normal and colon tumor cell lines. The results demonstrate that the CEA family of genes exists in a state of hypermethylation in normal cells. In contrast, the CEA gene(s) are relatively hypomethylated in the tumor cell lines, suggesting a correlation between the state of methylation and degree of expression of the CEA gene(s). Using a CEA cDNA probe, we have investigated the phenomenon of up-regulation of CEA expression in human tumor cell lines by recombinant human gamma-interferon (Hu-IFN- $\gamma$ ). An increase in MAb binding was shown to be accompanied by a 6- to 11-fold increase in the steady-state levels of three CEA transcripts with sizes of 4.2, 3.5, and 2.8 kb. Normal human fibroblasts had no detectable cytoplasmic CEA glycoprotein levels nor did they contain detectable levels of CEA mRNA, either before or after treatment with Hu-IFN- $\gamma$ . Our observations suggest that some common factors may also be involved in the regulation of the CEA and class II histocompatibility genes. Studies are also in progress to identify and characterize the gene(s) encoding the TAG-72 antigen.

### Major Findings

Using an assay based on the binding of a CEA-specific monoclonal antibody (MAB), we examined the expression of carcinoembryonic antigen (CEA) genes in human colon tumor and normal fibroblast cell lines. CEA expression was not detectable in the normal fibroblast cell lines, whereas varying levels of high CEA expression were found in the colon tumor cell lines LS-174T, GEO, and WiDr. We then used a 550-base pair CEA cDNA probe to carry out Southern analysis of the DNA isolated from the normal and colon tumor cell lines. At high stringency, the CEA probe detected seven *Bam*HI fragments in all DNAs analyzed. At low stringency, however, 14 *Bam*HI fragments ranging from 1.5 to 2.3 kb were detected. Results of the Southern analysis demonstrate no amplification or rearrangement of the CEA genes in tumor cells. We used methylation-sensitive restriction endonucleases, *Hpa*II and *Hha*I, to compare the degree of methylation of genes in the CEA family in normal and colon tumor cell lines. Our results demonstrate that the CEA family of genes exists in a state of hypermethylation in the normal cell lines. In contrast, the CEA gene(s) are relatively hypomethylated in the tumor cell lines, suggesting correlation between the state of methylation and degree of expression of the CEA gene(s). A comparison of the state of methylation of the CEA gene(s) in cells before and after treatment with recombinant human gamma-interferon (Hu-IFN- $\gamma$ ) showed no detectable difference in the degree of DNA methylation. The segments of CEA genes that are hypermethylated in normal cells, but are hypomethylated in tumor cells, were also identified. Thus, these studies may help identify the sites of methylation that are crucial for the control of CEA gene regulation.

The effect of Hu-IFN- $\gamma$  on the expression of human CEA and its related transcripts was then examined in several human colon carcinoma and normal human fibroblast cell lines. The colon tumor cell lines, HT-29, WiDr, and LS-174T, were each shown to express different constitutive levels of CEA glycopeptide as measured by the binding of the CEA-specific MAB COL-4. Treatment with Hu-IFN- $\gamma$  enhanced the level of binding of COL-4 in total cell extracts of HT-29 and WiDr cells by 2.5- and 6.5-fold, respectively. Using a CEA cDNA probe, this increase in MAB binding was shown to be accompanied by a 6- to 11-fold increase in the steady-state levels of three CEA transcripts with sizes of 4.2, 3.5, and 2.8 kb. On the other hand, Hu-IFN- $\gamma$  treatment had no effect on the level of COL-4 binding or expression of CEA transcripts in LS-174T colon carcinoma cells, which are high constitutive expressors of CEA glycoprotein. Normal human fibroblast cell lines MRC-5 and WI38 had no detectable cytoplasmic CEA glycopeptide levels nor did they contain detectable levels of CEA mRNA, either before or after treatment with Hu-IFN- $\gamma$ . In contrast, Hu-IFN- $\gamma$  induced the *de novo* expression of the normal major histocompatibility complex class II antigen, HLA-DR, on HT-29 and WiDr colon cancer cells as well as the two fibroblast cell lines. Treatment of the LS-174T cell line with Hu-IFN- $\gamma$  did not result in the induction of class II HLA-DR antigen. Our observations suggest that some common factors may be involved in the regulation of the CEA and class II histocompatibility genes. In addition, the demonstration that Hu-IFN- $\gamma$  enhances CEA expression in some carcinoma cell lines, but fails to induce *de novo* expression of CEA transcripts in fibroblasts, supports the potential application of Hu-IFN- $\gamma$  in enhancement of tumor targeting of anti-tumor MABs and adds to our understanding of the mechanism of IFN-mediated up-regulation of some tumor antigens.

*Publications*

Tran, R., Kashmiri, S.V.S., Kantor, J., Greiner, J.W., Pestka, S., Shively, J.E. and Schlom, J.: Correlation of DNA hypomethylation with expression of carcinoembryonic antigen in human colon carcinoma cells. *Cancer Res.* 48:5674-5679, 1988.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

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Parameters Involved in the Tumor Targeting of Monoclonal Antibodies

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

Patricia Horan-Hand	Research Chemist	LTIB, DCBD, NCI
Jean Simpson	Medical Staff Fellow	LTIB, DCBD, NCI
Alfredo Molinolo	Visiting Fellow	LTIB, DCBD, NCI
David Colcher	Supervisory Microbiologist	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. Andrew Raubitschek, Radiation Oncology Branch, National Cancer Institute  
 Dr. Philip Snoy, Center for Drugs and Biologics, Division of Product Quality Control, NIH

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Experimental Oncology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.7

## PROFESSIONAL:

0.6

## 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 unrounded type. Do not exceed the space provided.)

One of the problems observed in studies using radiolabeled monoclonal antibodies (MAbs) for detection of human tumors is the relatively low percent of the injected dose of MAb per gram of tumor. Recent studies in in vivo model systems for melanoma and hepatoma and clinical trials involving hepatoma patients have suggested enhanced deposition of radiolabeled antibody in tumors pretreated with external irradiation. One major goal of this project is to identify parameters that optimize binding of MAbs to carcinoma cells in vivo. We have initiated studies to determine the effect of carcinomas exposed to external irradiation prior to localization of tumors by MAB conjugates. These studies include evaluation of the effect of external irradiation on (a) growth of carcinoma xenografts, (b) cellular integrity and the expression of TAG-72 at the cellular level, and (c) binding of MAb B72.3 to carcinoma xenografts.

One approach to the treatment of patients with peritoneal carcinoma may be direct infusion into the peritoneal cavity of radiolabeled antibody conjugates. In comparison to intravenous MAB administration, this method may improve the percent of injected MAB dose per gram of tumor and may lead to less radio-labeled antibody in the bone marrow and therefore less toxicity to the patient. The second major goal of this project is to develop an in vivo model system to study immunotherapy of human carcinoma in the peritoneal cavity. These studies will include (a) determination of the animal species eliciting the pattern most comparable to that observed in the human of MAB clearance from the blood, (b) development of a human peritoneal carcinoma xenograft in an animal model, and (c) determination of the utility of intraperitoneally (i.p.) administered MAB conjugates for carcinoma therapy.



## MAJOR FINDINGS

The project on the identification of parameters that affect binding of MABs to tumor cells in vivo has led to studies of the effect of irradiation on MAB targeting of human carcinomas. As a model system we employed MAB B72.3 and the LS-174T human colon carcinoma xenograft in athymic mice. LS-174T tumors exposed to 300 cGy grew to approximately 93% the size of non-irradiated tumors, while those exposed to doses of 600-2000 cGy were approximately 41% the size of control tumors. Splitting the 900-cGy into three 300-cGy fractions yielded a two-fold lower tumor volume compared with a single 900-cGy fraction. Histochemical evaluation of the carcinomas revealed cellular changes consistent with early effects of radiation exposure. Expression of tumor-associated glycoprotein (TAG)-72, the high molecular weight mucin detected by MAB B72.3, was not affected by tumor irradiation. We chose a dose of 300 cGy for our initial experiments, to determine the effect of pre-irradiation on localization of MAB B72.3, since 300-cGy had the least cytotoxic effect on the LS-174T tumors and is more analogous to a clinical setting. Although we observed a trend toward enhanced MAB binding to tumor after irradiation, these results were not statistically significant. Significant effects were also not observed after pretreating tumors with 900 cGy as a single dose or in three 300-cGy exposures. Since one explanation for this may be that permeability changes caused by radiation are not sufficient to allow a significant difference in the ability of the 150,000 D IgG to cross the vascular epithelium, F(ab')<sub>2</sub> fragments (approximately 50,000 D) were also used for these studies and were found to provide no advantage over the whole IgG. Our results have shown, at most, an increase of 1.1- to 1.5-fold in MAB binding to tumor, however, these results were not statistically significant.

Our results suggest that consistent augmentation of radiolabeled antibody localization to tumors pretreated with external beam irradiation is not a universal phenomenon. Inherent differences in tumors, such as cell type of origin, size, spatial configuration, extent of vascularization, and volume of interstitial space, may contribute to variability of the effect of pre-irradiation of tumors on MAB localization. Extensive evaluation of each individual biological model is therefore required to determine the efficacy of novel therapeutic modalities.

Studies are now in progress to develop a reliable animal model system, which will allow assessment of the toxicity of i.p. MAB administration and provide pharmacokinetic data that may be successfully scaled up to man. The athymic (nu/nu) mouse bearing tumor xenografts is generally accepted as the animal model of choice for preclinical MAB studies. Our studies will investigate and compare the pharmacokinetics of plasma clearance of intravenous and i.p. administered MABs in athymic and Balb/c mice, athymic and Buffalo rats, cynomolgus and rhesus monkeys, and colorectal carcinoma patients. To determine if differences that may exist between MABs alter plasma clearance patterns, plasma clearance of MABs B72.3, COL-12 (anti-carcinoembryonic antigen), CC49, and CC83 (anti-TAG-72) will be compared. The effect of body surface areas, which are proportional to peritoneal surface areas, on i.p. MAB plasma clearance will be determined for each patient receiving MAB and the various animal models.

Anti-human tumor antibodies are unique because they react specifically with human tumor-associated antigens, such as carcinoembryonic antigen or TAG-72. No cross reactivity of these antibodies to rodent antigens is observed. For this reason, the model of choice for tumor targeting and therapy of MAbs vs. human tumor antigens is the athymic mouse bearing human tumor xenografts. Studies are also in progress to develop and use, as an in vivo model system for immunotherapy of human peritoneal carcinoma, i.p. xenografts of human ovarian carcinoma in athymic mice and MAb B72.3.

#### PUBLICATIONS:

Colcher D, Esteban J, Carrasquillo JA, Sugarbaker P, Reynolds JC, Bryant G, Larson SM, Schlom, J. Complementation of intracavitary and intravenous administration of a monoclonal antibody (B72.3) in patients with carcinoma. *Cancer Res.* 1987;47:4218-4224.

Shrivastav S, Schlom J, Raubitschek A, Molinolo A, Simpson J, Horan-Hand P. Studies concerning the effect of external irradiation on localization of radiolabeled monoclonal antibody B72.3 to human colon carcinoma xenografts. *Int. J. Radiat. Oncol. Biol. Phys.* 1989; 16:721-729.

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**NOTICE OF INTRAMURAL RESEARCH PROJECT**

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October 1, 1988 to September 30, 1989

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 and Tumorigenesis

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

Barbara Vonderhaar	Research Chemist	LTIB, DCBD, NCI
Suzanne E. Ziska	Senior Staff Fellow	LTIB, DCBD, NCI
Rina Das	Visiting Fellow	LTIB, DCBD, NCI
Erika Ginsburg	Biologist	LTIB, DCBD, NCI

COOPERATING UNITS (if any)

Dr. Rhoda Maneckjee, Medical Oncology Branch, NCI, Bethesda, MD; Dr. Sandra Haslam, Michigan State University, East Lansing, MI; Dr. Randy Witcomb, Massachusetts General Hospital, Boston, MA; Dr. Claudio Dati, University of Torino, Torino, ITALY

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

3.0

OTHER:

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 mammary gland is a complex organ whose growth and development is controlled by the interaction of a wide variety of hormones and growth factors. These same factors play fundamental roles in the etiology and progression of the cancerous state. The first event in the action of these hormones and growth factors is the interaction with specific cell associated receptors. The availability and activity of each class of receptor is regulated by the ligand which it recognizes as well as the general hormonal/growth factor milieu of the target cell. Our emphasis has been on the interactions of prolactin, thyroid hormone, and estrogens with recent work also examining how epidermal growth factor (EGF), and EGF-like growth factors are affected by the interplay of these three classical hormones. In addition we have explored the relationship of membrane associated antiestrogen binding sites to the lactogenic hormone receptor and the action of platelet derived growth factor (PDGF) on human breast cancer cell growth in culture. T47D cells have specific PDGF receptors and respond to its growth promoting signal by inducing phosphorylation of a 65Kd protein in the calelectrin family. Lobulo-alveolar development of the mammary gland requires the priming action of both estrogen and progesterone to induce EGF receptors and production of EGF-like growth factors. In concert with insulin, prolactin and glucocorticoids, EGF or  $\alpha$ -TGF can promote full lobulo-alveolar development *in vitro*. The primed mammary gland is more sensitive to  $\alpha$ -TGF than to EGF. Growth promotion of MCF-7 human breast cancer cells by prolactin can occur in the complete absence of estrogens. Prolactin induced growth of the estrogen receptor negative Nb2 rat lymphoma cell can be blocked by non-steroidal antiestrogens such as tamoxifen. This action is through the membranous antiestrogen binding site (AEBS) which may be intimately associated with the prolactin receptor. The antiprolactin action of tamoxifen, working through the AEBS, may have important clinical implications.

related non-steroidal, triphenylethylene antiestrogens with an apparent  $K_d$  of  $3.1 \times 10^{-10}M$ . The AEBS do not bind estrogens. These sites are distinct from estrogen receptors. Antiestrogens act through the AEBS to inhibit the growth promoting effect of Prl on the Nb2 cells. Antiestrogens of the class which bind to the AEBS also inhibit the binding of Prl to its receptor. The order of affinities of the various antiestrogens for the AEBS parallels the order of their potencies as growth and lactogen binding inhibitors. They do not affect the binding of other ligands to their membrane associated receptors. Thus, in addition to their well defined antiestrogenic actions, these agents may be antilactogenic as well. Future work will center on assessing the AEBS in normal mammary glands and in the Prl responsive breast cancer cells (MCF-7, T47D and ZR-75-1) to determine if they (a) are present, (b) inhibit Prl binding to its receptor, and (c) block the action of Prl on milk protein synthesis (normal) and cell growth (breast cancer cells). We will continue the characterization of the Prl receptor isolated by affinity chromatography and immunopurification and determine what, if any, physical relationship exists between this receptor and the AEBS.

#### Publications:

Vonderhaar, B.K. Regulation of development of the normal mammary gland by hormones and growth factors. In: Lippman, M.E., Dickson, R., eds. Breast Cancer: Cellular and Molecular Biology. New York: Martinus Nijhoff, 1988, 251-66.

Ziska, S.E., Bhattacharjee, M., Herber, R.L., Qasba, P.K. and Vonderhaar, B.K.: Thyroid hormone regulation of alpha-lactalbumin: Differential glycosylation and mRNA synthesis in mouse mammary glands. *Endocrinology* 123: 2242-2248.

Vonderhaar, B.K., and Ziska, S.E., Hormonal regulation of milk protein gene expression. *Annual Rev. Physiol.* 1989, 51: 641-52.

Biswas, R. and Vonderhaar, B.K.: Antiestrogen inhibition of prolactin induced growth of the Nb2 rat lymphoma cell line. *Cancer Research*, 1989, in press.

Vonderhaar, B.K.: Prolactin induced growth of MCF-7 human breast cancer cells in the absence of estrogens. *Cancer Letters*, 1989, in press.



### Major Findings:

In addition to the classical hormones such as insulin (I), corticoids (A and H), prolactin (Prl), estrogen (E) and thyroid hormone (T<sub>3</sub>), it is becoming increasingly clear that a variety of growth factors are involved in the development and differentiation of the mammary gland in both an autocrine and a paracrine manner. Platelet derived growth factor (PDGF) which is found in milk and is produced by human breast cancer cells in culture has been reported to act in breast tissue only in a paracrine manner. However we have now shown that specific, high affinity PDGF receptors are present on the membranes of T47D cells grown in culture in the presence of 0.5% charcoal stripped serum (CSS) or platelet poor plasma (PPP). In addition, the cells respond to the growth stimulating signal of PDGF with a 2 fold increase in cell number within 3 days of culture. The maximal response occurs with 1.25 half-maximal units of PDGF (125ng/ml). Within 7 min. of addition of PDGF to cultures of the T47D cells, specific phosphorylation of a 65Kd protein in the calelectrin family occurs. Thus it appears that PDGF may be an autocrine growth factor for breast epithelial cells as well.

Lobulo-alveolar development of the mammary gland is also under the control of growth factors in the epidermal growth factor (EGF) family. Previously we had shown that the in vitro induction of lobulo-alveolar development in mouse mammary glands under the influence of the hormones I, Prl, A and H also required the presence of EGF in order to occur. We have now shown that  $\alpha$ -transforming growth factor ( $\alpha$ -TGF) is fully active in promoting lobulo-alveolar development in vitro. Full activity (>90% of the glands responding) was achieved with EGF at 60ng/ml but only 30% of the glands responded with 20ng EGF/ml. In contrast,  $\alpha$ -TGF gave complete response at both high and low doses (60 and 6 ng EGF equivalents/ml). This greater sensitivity of the glands to  $\alpha$ -TGF in vitro is in complete agreement with our previously published observations in vivo using growth factors locally implanted into mammary glands of developing mice.

Glandular development occurs in vitro only after priming with estrogen and progesterone (E/P). This priming increases the ability of the mammary tissue to bind the growth factor as well as induces the production of a mammary derived EGF-like growth factor. Estrogen (E) alone or progesterone (P) alone is not sufficient during the priming process nor can the addition of E and P to the culture medium overcome the need for priming in vivo. Priming by E/P cannot be replaced by EGF priming (either using EGF directly or by elevating salivary gland production of EGF with testosterone). Injecting the animals with anti-EGF during the priming with E/P does not prevent the priming process. All of these data suggest that the role of E and P in priming is a complex one possibly involving positive as well as negative actions by these hormones. Future work will examine the role of each of these hormones in the induction of the growth factor receptors as well as the induction of the EGF-like growth factor using ovariectomized mice. The characteristics of binding of both EGF and  $\alpha$ -TGF to breast tissue will be examined in detail to determine whether the differences in sensitivity to the two related growth factors lies with the receptor or with post-receptor events. In addition, the possibility that the priming removes a natural inhibitor of lobulo-alveolar development (i.e.  $\beta$ -TGF) will be examined.

Prolactin/thyroid hormone/estrogen interactions were explored at the level of differentiation as well as growth regulation. The action of Prl in milk protein production is at the transcriptional level. Physiological levels of T<sub>3</sub>, in the presence of Prl, increase transcription and promote differential glycosylation of  $\alpha$ -lactalbumin resulting in its increased secretion. Under the proper conditions of serum staging, Prl promotes growth of several human breast cancer cell lines including MCF-7, ZR-75-1 and T47D. The Prl effect occurs in the complete absence of estrogens (i.e. in phenol red free medium). Prl also promotes the growth of the Nb2 cells in the absence of estrogens. Serum free conditions for the growth of the Nb2 cells have been developed which enhance the sensitivity of the cells to Prl. The Nb2 cell is estrogen receptor negative. The Nb2 cell does contain membrane associated antiestrogen binding sites (AEBS) which bind tamoxifen and

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 CB 09025-02 LT1B

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Antibody Directed Cellular Immunotherapy of Colon Cancer

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

F. James Primus	Cancer Expert	LT1B, DCBD, NCI
Tribhuvan K. Pendurthi	Guest Researcher	LT1B, DCBD, NCI
Jeffrey Schlom	Chief	LT1B, DCBD, NCI

COOPERATING UNITS (if any)

Dr. David Segal, Immunology Branch, DCBD, NCI, NIH; Dr. Michael Lotze, Surgery Branch, DCT, NCI, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.1

PROFESSIONAL:

2.1

OTHER:

0

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

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

This project is examining the role that monoclonal antibodies (MAB) against human colonic tumor antigens have in directing and augmenting the cytotoxic activity of NK/LAK cells and T cells. A MAB designated D612 (IgG2a) has been generated that uniformly reacted with the cell surface of approximately 80% of primary or metastatic colorectal carcinomas. Normal tissue reactivity of D612 was confined mainly to the small and large intestine, while non-gastrointestinal normal tissues were routinely negative. D612 mediated antibody-dependent cellular cytotoxicity in conjunction with normal human peripheral blood mononuclear cells was increased by effector cell activation with interleukin 2 (IL-2). For retargeting of T cells, heteroantibody conjugates between the anti-colon tumor MAB, B38.1, and anti-CD3 (OKT-3) were chemically prepared, and these were tested along with effectors obtained from PBMNC stimulated with anti-CD3 and IL-2. The heteroantibody-mediated cellular cytotoxicity activity was similar on a per cell basis among effector populations prepared by stimulation with IL-2, anti-CD3, or a combination of these reagents. However, the total lytic activity of anti-CD3 + IL-2-stimulated cultures was much higher due to the greater expansion in cell number achieved by the combination treatment. These studies suggest that MABs with restricted normal tissue reactivity and ability to direct the cellular cytotoxicity of a broader spectrum of killer lymphocytes activated by lymphokines and other agents might augment the efficacy of colon cancer immunotherapy.

### Major Findings

A monoclonal antibody (MAB), designated D612 (IgG2a), was generated by immunization of mice with a membrane-enriched fraction of a moderately differentiated primary colon adenocarcinoma. Using membrane extracts derived from a variety of cell lines and normal and neoplastic tissues, D612 was found to react specifically with extracts of cells or tissues originating from the large intestine. The D612 antigen was not detected by immunohistochemical staining of conventionally processed tissue specimens, but was revealed when fresh, frozen tissues were used. An immunohistochemical survey of the tissue distribution of the D612 antigen showed that 70 to 80% of primary or metastatic colorectal carcinomas were positive, independent of their differentiation. In over half of these specimens, the staining was homogeneously distributed among the majority of tumor cells where it localized predominantly to the plasma membrane. Flow cytometry of colon tumor cell lines also showed that the D612 antigen occupied a surface location. D612 did not stain noncarcinoma tumors but did react weakly with a small number of stomach, breast, and ovarian carcinomas in a highly focal pattern. Normal tissue reactivity of D612 was confined to the epithelium of the small and large intestine and of the stomach. It was not found in normal specimens covering a wide range of nongastrointestinal tissues including thyroid, kidney, pancreas, liver, bladder, blood cells, and lung. Normal colon goblet and absorptive cells showed a homogeneous pattern of staining, located primarily along the basolateral cytoplasmic membrane. Other studies showed that the D612 antigen is not secreted into the culture media, has a molecular size of  $1 \times 10^6$  or greater, and is not CEA, TAG, or the 17-1A antigen.

The D612 MAB was found to mediate antibody-dependent cellular cytotoxicity (ADCC) in conjunction with normal human peripheral blood mononuclear cells (PBMC) against antigen-positive colon tumor cell lines. Exposure of PBMC to IL-2 (100 U/ml; 24 hr) resulted in a 2- to 3-fold increase in specific ADCC cytolytic activity. Although the total specific ADCC lytic activity varied among different donors, its potentiation by IL-2 was very similar. The ADCC activity of the 17-1A MAB was also studied for comparison to D612, and it was found that both MABs were similar in their ability to mediate ADCC. The association and dissociation constants of these two MABs were measured using radiolabeled MAB and the LS-174T colon tumor cell line. For D612, the  $K_a$  and  $K_d$  were calculated to be  $1.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  and  $8.5 \times 10^{-4} \text{ s}^{-1}$ , respectively. The corresponding values for the 17-1A MAB equaled  $4.4 \times 10^7$  and  $9.3 \times 10^{-4}$ , respectively. The number of sites per LS-174T cell was found to be  $4.8 \times 10^5$  for D612 and  $1.1 \times 10^6$  for 17-1A. It can be seen from these studies that the D612 and 17-1A MABs have equivalent effector functional activity while the D612 MAB has a better association constant.

Taken together, the tissue distribution, cytological location, and immunochemical characteristics of the antigen recognized by D612 indicate that the D612 MAB is different from a large variety of anti-colon carcinoma MABs previously described in the literature. The D612 antigen has a fairly uniform expression within and among colorectal carcinomas, restricted normal tissue reactivity, and cell surface location--desirable properties for an antigen intended to serve as a target for immunotherapy. Furthermore, radiolabeled D612 was shown to localize colonic tumor xenografts. Its ability to mediate ADCC coupled with IL-2 augmentation of this property indicate that further studies of antibody-dependent cellular immunotherapy with this MAB would be worthwhile.

Retargeting of T-cell experiments were initiated using a MAB, designated B38.1 (IgG1), that was generated by immunization with membrane-enriched fractions of



human metastatic mammary carcinoma cells. Cross-competition experiments between B38.1, 17-1A, and a second generation MAb, designated GA73.3, showed that these MAbs cross-blocked one another, with the B38.1 and GA73.3 pair showing identical activities. Furthermore, B38.1 immunoprecipitated three bands, from an intrinsically labeled colon carcinoma cell line, that migrated in the range of 37 to 40 kDa, similar to the profile revealed by GA73.3 immunoprecipitates. The tissue distribution properties of B38.1, and for comparison, the GA73.3 MAB, were analyzed immunohistochemically with fresh, frozen specimens. Both MAbs reacted with a variety of normal tissues, such as thyroid, liver, kidney, pancreas, and colon, with a similar staining pattern. Collectively, these results indicate that the B38.1 MAB most likely recognizes the same antigen detected by 17-1A/GA73.3.

Heteroconjugates were prepared between B38.1 and OKT-3 (anti-CD3) by disulfide linkage using the heterobifunctional reagent SPDP. Heteroantibody preparations were made with intact MAb or F(ab')<sub>2</sub> fragments, and an essentially dimeric heteroantibody fraction was then isolated by molecular sieve chromatography. Heteroantibody-mediated cellular cytotoxicity (HMCC) against cultured colon carcinoma cell lines was measured in short-term chromium release assay after activation of normal human PBMC with solid-phase OKT-3 followed by incubation with rIL-2 (100 U/ml). Monomeric B38.1 variably mediated low levels of ADCC with PBMC activated with IL-2 alone, an expected finding based on the IgG1 isotype of B38.1, but was without activity in conjunction with effector cells exposed to anti-CD3 + IL-2. Heteroantibodies formed with intact Ig or F(ab')<sub>2</sub> fragments were equally effective in retargeting effector cells activated with anti-CD3 alone, or with anti-CD3 + IL-2. Depletion of FcR III-positive lymphoid cells before activation did not alter heteroantibody retargeting, demonstrating that natural killer cells were not the precursors for the effector population activated in this manner for HMCC. HMCC activity with effector cells produced by exposure to anti-CD3 and IL-2 was similar on a per cell basis to that of cells incubated with anti-CD3 or IL-2 alone. However, greater total HMCC activity was obtained with PBMC cultures treated with anti-CD3 + IL-2, since this combination resulted in a 30- to 50-fold increase in cell number after 9 days of IL-2 as compared to a doubling of cell number in cultures exposed to anti-CD3 or IL-2 alone. These studies suggest that lymphoid cells, probably T cells for the most part, activated by anti-CD3 and IL-2 may be alternative candidates for immunotherapy when used in conjunction with retargeting heteroantibodies.



*Publications*

1. Muraro, R., Nuti, M., Natali, P.B., Bigotti, A., Simpson, J.F., Primus, F.J., Colcher, D., Greiner, J.W., and Schlom, J.: A monoclonal antibody (D612) with selective reactivity for malignant and normal gastrointestinal epithelium. *Int. J. Cancer* 1989, in press.
2. Kuroki, M., Greiner, J.W., Simpson, J.F., Primus, F.J., Guadagni, F., and Schlom, J.: Serologic mapping and biochemical characterization of the carcinoembryonic antigen epitopes using fourteen distinct monoclonal antibodies. *Int. J. Cancer* 1989, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09026-02 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

## Characterization of Human Tumor-Associated Antigens

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

Philip D. Fernsten	Expert	LTIB, DCBD, NCI
Raju Katari	BPT Fellow	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. Robert Raubitschek, Radiation Oncology Branch, Division of Cancer Treatment, NCI

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Experimental Oncology

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

2.5

## PROFESSIONAL:

2.1

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Human adenocarcinoma cells secrete and express on their cell surfaces high-molecular-weight, mucin-like glycoproteins that bear epitopes that are highly restricted to carcinoma cells. Monoclonal antibody B72.3, which has been extensively characterized as to reactivity with a variety of carcinomas versus normal tissues, reacts with such a mucin-like glycoprotein. The B72.3-reactive antigen, designated TAG (tumor-associated glycoprotein)-72, was partially purified and used as immunogen to produce 18 second generation anti-TAG-72 antibodies, designated CC antibodies. Based on competitive binding studies, a number of the epitopes recognized by the anti-TAG-72 antibodies appear to be structurally or spatially related, but none appear to be identical. One of the second generation antibodies was selected to develop a procedure to purify preparative amounts of TAG-72. TAG-72, extracted from xenografted human colon carcinoma cells, was subjected to antibody-affinity, size-exclusion, and ion-exchange chromatography. A double determinant radioimmunoassay revealed a greater than 1000-fold purification with a greater than 10% yield. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of radiolabeled purified TAG-72 revealed a polydisperse, but apparently homogenous, high-molecular-weight glycoprotein. The density of purified TAG-72 was 1.45 g/ml, indicating a high degree of glycosylation. Amino acid analysis of the purified TAG-72 revealed a profile highly similar to that reported for other purified mucins. The results of double immunodiffusion assays indicate that each epitope recognized by the anti-TAG-72 antibodies is expressed in multiple copies on the TAG-72 molecule. The purified TAG-72 has been partially deglycosylated with trifluoromethane sulfonic acid in preparation for amino acid sequence analysis. Other studies are underway to determine the physical location, coexpression, and chemical makeup of the various tumor-specific epitopes borne on the purified TAG-72; to analyze and compare TAG-72 derived from other sources, including other human solid tumors, pleural effusions, ascites fluids, serum, etc., with that derived from LS174T xenografts; and to compare the spectrum of expression of the various tumor-specific epitopes on TAG-72 derived from various sources.

### Major Findings

Xenografts of the LS174T human colon carcinoma cell line have been shown in a double determinant radioimmunoassay to contain levels of the B72.3-reactive antigen, tumor-associated glycoprotein (TAG)-72, equivalent to the highest levels found in primary and metastatic human colon and breast carcinomas. Western blotting analyses of LS174T extracts revealed that the antigen had a molecular size distribution highly similar to that of TAG-72 derived from human primary and metastatic breast and colon carcinomas. TAG-72 was partially purified from extracts of LS174T xenografts by gel filtration on Sepharose CL-4B followed by B72.3 antibody-affinity chromatography, and used as immunogen to produce 19 second generation anti-TAG-72 monoclonal antibodies (MAbs), designated CC antibodies. Based on competitive binding studies, a number of the epitopes recognized by the anti-TAG-72 MAbs appear to be structurally or spatially related, but none appear to be identical. All of the epitopes are sensitive to mild periodate oxidation and 15 of 19 are sensitive or partially sensitive to neuraminidase digestion. All of the anti-TAG-72 antibodies produced immune precipitate lines with purified TAG-72 in double immunodiffusion assays, indicating that each epitope recognized by the antibodies is expressed in multiple copies on the TAG-72 molecule.

Due to the high levels of TAG-72 found in LS174T xenografts as well as its relative ease of availability, LS174T xenografts were also chosen as the starting material for purification of preparative amounts of TAG-72. CC49, one of the second generation antibodies elicited by partially purified TAG-72, was selected for utilization in a larger scale purification scheme because of its higher affinity of binding to TAG-72 than MAb B72.3. LS174T xenografts were homogenized in a non-ionic detergent and centrifuged at  $10,000 \times g$  and  $100,000 \times g$ . Because TAG-72 is both heat and acid stable, the supernatants were then either extracted at  $100^\circ\text{C}$  or with perchloric acid. Following cooling or neutralization, the supernatants were centrifuged again, reduced with dithiothreitol, and alkylated with iodoacetamide. This material was then applied to a CC49 affinity column. After washing and elution with 6 M guanidine HCl, the affinity-purified TAG-72 was separated from the bulk of the remaining protein on a Superose 6 column equilibrated with 6 M guanidine HCl. Final purification was achieved using a DEAE-5PW column equilibrated with 8 M urea and 20 mM formic acid, pH 3.5. Under these conditions, the remaining contaminating protein failed to bind to the column matrix, and the TAG-72 was recovered by elution with NaCl. A purification of greater than 1000-fold with a total yield of greater than 10%, as determined by amino acid analyses and double determinant radioimmunoassays, was achieved using this protocol. Autoradiographs of radioiodinated purified TAG-72 separated on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) showed a high-molecular-weight polydisperse, but apparently homogenous, band. Similar results were obtained by periodic acid-Schiff's reagent staining or Western blotting analyses using CC49 immunostaining of purified TAG-72 run on SDS-PAGE. The purified TAG-72 preparations did not contain material that stained with Coomassie blue. Amino acid analyses of the purified TAG-72 revealed a composition highly enriched in serine, threonine, glycine, proline, and glutamine and glutamate and containing only trace amounts of methionine, tyrosine, and phenylalanine. This amino acid profile is highly consistent with that reported for other purified mucins. The purified TAG-72 has been subjected to partial deglycosylation using trifluoromethane sulfonic acid for 8 hours at  $4^\circ\text{C}$ . Discrete peptides generated from this material will be subjected to amino acid sequence analysis.

*Publications*

Muraro, R., Kuroki, M., Wunderlich, D., Poole, D.J., Colcher, D., Thor, A., Greiner, J.W., Simpson, J.F., Molinolo, A., Noguchi, P., and Schlom, J.: Generation and characterization of B72.3 second generation monoclonal antibodies reactive with the tumor-associated glycoprotein 72 antigen. *Cancer Res.* 1988; 48:4588-4596.

Sheer, D.G., Schlom, J., and Cooper, H.L.: Purification and composition of the human tumor-associated glycoprotein (TAG-72) defined by monoclonal antibodies CC49 and B72.3. *Cancer Res.* 1988; 48:6811-6818.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09006-07 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Studies on the Nature and Function of the Phosphoprotein, Prosolin

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

Herbert L. Cooper

Chief, Cell &amp; Molec.

LTIB, DCBD, NCI

Physiol. Section

Rebecca Fuldner

Biotechnology Research Fellow

LTIB, DCBD, NCI

Richard Braverman

Chemist

LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Cellular &amp; Molecular Physiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

2.5

## OTHER:

0.5

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

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

We have extensively characterized prosolin, a major cytosolic protein of proliferating HL-60 promyelocytic leukemia cells that undergoes very rapid phosphorylation in response to treatment of the cells with tumor-promoting phorbol esters like TPA as an early step in a pathway leading to cessation of cell growth and onset of monocytoïd differentiation. Prosolin has now been identified in proliferating human peripheral blood lymphocytes (PBL) by tryptic peptide mapping of proteins isolated from two-dimensional electrophoretic gels. While virtually absent from resting PBL, prosolin expression is induced by mitogens as a late event, during the onset of S-phase. Its level of synthesis correlates with the prevalent level of DNA synthesis. Prosolin undergoes rapid phosphorylation in proliferating PBL in response to TPA treatment, together with an inhibition of DNA synthesis. Phosphorylation of prosolin may be part of the mechanism by which naturally occurring growth inhibitory factors terminate normal lymphocyte proliferation. T-cell leukemia cell lines show a marked reduction in the rapidity of the phosphorylation of prosolin in response to TPA and other agents. The degree of reduction of the TPA-induced phosphorylation of prosolin in T-cell leukemias is related to the degree of undifferentiation of the neoplastic cells. We are cloning and sequencing cDNAs to prosolin. Partial amino acid sequence data were obtained by analysis of tryptic peptides of prosolin isolated from two-dimensional electrophoretic gels. Degenerate oligonucleotide probes were synthesized and used to probe a lymphoblast cDNA library. Positive clones are currently being analyzed.

### *Major Findings*

Prosolin is a major cytosolic protein of proliferating HL-60 promyelocytic leukemia cells that undergoes very rapid phosphorylation in response to treatment of the cells with tumor-promoting phorbol esters like TPA. We have characterized this protein extensively and have shown that its TPA-induced phosphorylation is an early step in a pathway leading to cessation of cell growth and onset of monocytoid differentiation in HL-60 cells.

Prosolin has now been identified in proliferating human peripheral blood lymphocytes (PBL) by tryptic peptide mapping of proteins isolated from two-dimensional electrophoretic gels. While virtually absent from resting PBL, prosolin expression is induced by mitogens as a relatively late event, during the onset of S-phase. Once induced in rapidly proliferating lymphocytes, prosolin becomes a major cytosolic protein and its level of synthesis correlates with the prevalent level of DNA synthesis. As in HL-60, prosolin undergoes rapid phosphorylation in proliferating PBL in response to TPA treatment, an effect which correlates with an inhibition of DNA synthesis. Thus, prosolin may play a necessary role in the S-phase of the cell cycle, and its phosphorylation may be part of the mechanism by which lymphocytes exit from S-phase and suspend proliferation. We are exploring the possibility that phosphorylation of prosolin may be part of the mechanism by which naturally occurring growth inhibitory factors terminate normal lymphocyte proliferation.

We have found that T-cell leukemia cell lines show a marked reduction in the rapidity of the phosphorylation of prosolin in response to TPA and other agents. This may provide a clue to the biochemical basis for their failure to respond to normal signals that terminate lymphocyte proliferation. Results to date suggest that the degree of reduction of the TPA-induced phosphorylation of prosolin in T-cell leukemias is related to the degree of undifferentiation of the neoplastic cells.

We are currently cloning and sequencing cDNAs to prosolin. Partial amino acid sequence data were obtained by analysis of tryptic peptides of prosolin isolated from two-dimensional electrophoretic gels. Degenerate oligonucleotide probes were synthesized and used to probe a lymphoblast cDNA library. Positive clones are currently being analyzed. Deduced amino acid sequence information may allow us to compare the structure of prosolin with that of other proteins so as to improve our understanding of its function and the control of its phosphorylation. Full length cDNAs to prosolin will facilitate Northern and Southern blot analysis of prosolin expression and genomic organization and will permit production of recombinant prosolin. This will allow us to produce monoclonal antibodies, to conduct detailed phosphorylation studies, and to perform biochemical and molecular biological experiments on the function of prosolin.

We are also investigating the role of  $Ca^{2+}$  fluxes in prosolin phosphorylation and lymphocyte growth regulation.  $Ca^{2+}$  ionophore treatment of PBL results in extensive phosphorylation of prosolin and production of a series of putative phosphoprosolin forms not previously seen in TPA-treated cells. Characterization of these multiple phosphorylated forms of prosolin is in progress.

Longer term projects in this area include more precise examination of the cell-cycle specificity of the synthesis and phosphorylation of prosolin, and attempts to clarify its function by studying its interactions, *in vivo* and *in vitro*, with other molecules. Finally, the possible diagnostic and prognostic significance of the reduced phosphorylation response of prosolin to TPA and other agents in lymphoid leukemia cells will be explored.

*Publications*

Cooper, H.L., and Braverman, R. Phosphorylation of prosolin and leukocyte growth regulation. In: *Lymphocyte Activation and Differentiation*, Mani, J.C., and Dornand, J. (Eds.), Berlin, DeGruyter & Co., 1988, pp. 67-72.

Cooper, H.L., McDuffie, E., and Braverman, R. Human peripheral lymphocyte growth regulation and response to phorbol esters is linked to synthesis and phosphorylation of the cytosolic protein, prosolin. Submitted for publication.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09022-03 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Cytoskeletal proteins in oncogenic transformation and human neoplasia

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

Herbert L. Cooper

Chief, Cell. &amp; Molec.

LTIB, DCBD, NCI

Physiol. Section

G. L. Prasad

Visiting Fellow

LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Eva M. Valverius, Georgetown University Hospital, Washington, DC; David S. Salomon, LTIB

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Cellular &amp; Molecular Physiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

 (a) Human subjects (b) Human tissues (c) Neither (a1) Minors

B

 (a2) Interviews

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

We are examining the relationship between neoplastic transformation and biochemical derangements of expression or utilization of tropomyosins (TMs). In NIH/3T3 fibroblasts transformed by a number of related and unrelated retroviral oncogenes, expression of the transformed phenotype was reproducibly correlated with suppression of synthesis of two specific TM isoforms ("muscle type"). Of all the proteins detectable on two-dimensional gel electrophoresis of whole-cell proteins, only the TMs behaved in this way. Treatment of rat fibroblasts with tumor growth factor- $\alpha$  (TGF- $\alpha$ ), in addition to inducing the transformed phenotype, specifically suppressed synthesis of muscle-type TMs and inhibited their utilization in the cytoskeleton, strengthening the connection between suppression of TM and expression of the transformed phenotype and adding support at the biochemical level for an autocrine pathway in oncogene action. The findings point out the close biochemical relationship between epidermal growth factor (EGF) receptor activation and microfilament organization. Tropomyosin expression in relation to oncogene expression and neoplasia is being studied in murine and human epithelial cell systems. In mouse mammary epithelial cells transformed by the Ha-ras oncogene, the ratio of TM to actin accumulating in the cytoskeleton was reduced, indicating the production of microfilaments deficient in TM. The TM isoforms expressed by normal diploid human mammary epithelial cells have been compared with the pattern expressed by breast carcinoma cell lines. Defects in expression of particular TM isoforms occurred in all of the breast carcinoma cell lines studied, suggesting a possible role of deranged TM expression in human neoplasia.



### Major Findings

We are examining the relationship between neoplastic transformation and biochemical derangements of expression or utilization of tropomyosins (TM), a family of microfilament-associated proteins now known to occur in all cell types. TMs are an essential component of microfilaments, present in a strict stoichiometric relationship to the major microfilament protein, actin. In a study of NIH/3T3 fibroblasts transformed by a number of related and unrelated retroviral oncogenes, we found that expression of the transformed phenotype was reproducibly correlated with suppression of synthesis of two specific TM isoforms ("muscle type"). Of all the proteins detectable on two-dimensional gel electrophoresis of whole-cell proteins, only the TMs behaved in this way. Similar observations were made with NIH/3T3 fibroblasts transformed by chemical mutagens.

Transformed fibroblasts are known to elaborate transforming growth factors, particularly TGF- $\alpha$ , which has been proposed as a mechanism of transformation utilizing an autocrine loop. We found that treatment of rat fibroblasts with TGF- $\alpha$ , in addition to inducing the transformed phenotype, specifically suppressed synthesis of muscle-type TMs and inhibited their utilization in the cytoskeleton. This further strengthened the connection between suppression of TM and expression of the transformed phenotype and added support at the biochemical level for an autocrine pathway in oncogene action. The findings point out the close biochemical relationship between EGF receptor activation and microfilament organization.

Since most human malignancies occur in cells of epithelial rather than fibroblast lineage, we examined a murine epithelial cell model system. Immortalized "normal" mouse mammary epithelial cells transformed by the Ha-ras oncogene showed no reduction in TM synthesis. However, the synthesis of actin was increased, and the ratio of TM to actin accumulating in the cytoskeleton was reduced, indicating the production of microfilaments deficient in TM. Thus, TM deficiency in microfilaments may be the disturbance of TM metabolism that is common to transformation in both fibroblasts and epithelial cells.

The relevance of disturbed TM expression to human carcinoma is being studied in cultured lines derived from human breast carcinomas. The array of TM isoforms expressed by normal diploid human mammary epithelial cells has been characterized and compared with the pattern expressed by cell lines derived from breast carcinoma. Defects in expression of particular TM isoforms, homologous to the muscle-type TMs suppressed in transformed fibroblasts, occurred in all of the breast carcinoma cell lines studied. We plan to extend these studies to fresh specimens of normal and neoplastic breast tissue to determine whether such defects occur in tumors as well as in established tumor cell cultures. Other studies will examine the effect of transforming oncogenes on TM expression in mammary epithelial cells to determine if defects observed in breast cancer tissues and cell lines resemble those induced by transforming oncogenes. This will help clarify the question of the prevalence of oncogene-related mechanisms in carcinogenesis.

In related work, we are developing cDNA clones specific for the TMs expressed by epithelial cells. These will serve as probes in studies of expression of the various TM isoforms and in elucidating the mechanisms controlling them. They will also be used in Southern blot analyses of DNAs from breast cancer cells to determine whether deletion or rearrangement of TM genes is responsible for altered expression of particular isoforms. Together with the preceding study,

the information to be obtained will furnish perspective on the question of the relative importance of mutagenic versus oncogene-related processes in carcinogenesis.

To determine whether disturbed TM expression plays a causal role in transformation, we have begun a study aimed at specifically suppressing TM synthesis by the use of genetic insertions that produce antisense RNA sequences to specific TM transcripts. The initial stages of this study are in progress. Oligodeoxynucleotides homologous to the initiation region of a muscle-type TM isoform have been synthesized, ligated in both sense and antisense producing orientations into an expression vector with a selectable neomycin (G418) resistance marker, and transfected into normal rat kidney fibroblasts. Resistant cell clones have been selected and are currently being analyzed. The converse of this approach--insertion of a construct to restore TM production in order to reverse transformation--is being planned.

*Publications*

Cooper, H.L., Bhattacharya, B., Bassin, R.H., and Salomon, D.S. Suppression of synthesis and utilization of tropomyosin in mouse and rat fibroblasts by alpha-transforming growth factor. *Cancer Res.* 1987;47:4493-4500.

Bhattacharya, B., Ciardiello, F., Salomon, D.S., and Cooper, H.L. Disordered metabolism of microfilament proteins, tropomyosin and actin, in mouse mammary epithelial cells expressing the Ha-ras oncogene. *Oncogene Res.* 1988;3:51-65.

Sheer, D.G., Schlom, J., and Cooper, H.L. Purification and composition of the human tumor-associated glycoprotein (TAG-72) defined by monoclonal antibodies CC49 and B72.3. *Cancer Res.* 1988;48:6811-6818.

Bhattacharya, B., Prasad, G.L., Valv rius, E.M., Salomon, D.S., and Cooper, H.L. Tropomyosins of human mammary epithelial cells: consistent defects of expression in mammary carcinoma cell lines. Submitted for publication.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04848-17LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

"Anti-oncogenes": The analysis of cellular resistance to transformation

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

R. Bassin	Chief, Biochemistry of Oncogenes Sect.	LTIB, DCBD, NCI
B. Blondel	Visiting Fellow	LTIB, DCBD, NCI
D. Salomon	Research Biologist	LTIB, DCBD, NCI
R. Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI
N. Talbot	Biochemistry of Oncogenes Section	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. Donald Blair, Chief, Microbiology Section, LMO, DCCP, NCI;  
 Dr. Len Benade, Head, Virus Group, American Red Cross, Rockville, MD; Dr. Arie Moran, Ben Gurion University of the Negev, Israel; Dr. Makoto Noda, Riken Institute of Science, Japan; Dr. Yanagihara, Hiroshima University, Japan

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Biochemistry of Oncogenes

## INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.2

## PROFESSIONAL:

3.2

## OTHER:

2.0

## CHECK APPROPRIATE BOXES)

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

B

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

We have developed a new technique for isolating cells that resist transformation by specific oncogenes using 4-*cis*-hydroxy-L-proline as a selective agent. We have begun to characterize additional revertants from *ras*-transformed cells. The new revertants are resistant to transformation by members of the *ras* gene family and to certain tyrosine kinase oncogenes, but they are sensitive to retransformation by *mos*, *raf*, and *fos*. Combining the retransformation data observed here with those from our ouabain-derived revertants isolated previously (C-11 and F-2), we find that all oncogenes can be placed into one of four groups, and we suggest that this represents a primitive functional classification. If true, the *ras* gene would require the role of at least two cellular 2 functions (such as attachment to the cell membrane and kinase activity) to successfully transform mouse cells.

In another series of experiments, 3 of 10 otherwise inactive human tumor DNA samples tested positive for focus-forming activity when used to transfect indicator cells incubated without serum. Cells infected with the mouse *c-myc* gene in a retroviral vector also efficiently transform NIH/3T3 clone 7 cells in the absence of serum. The inhibition of focus-forming activity of *c-myc* appears to take place after the synthesis of *myc*-specific mRNA. The nature of the inhibitor present in serum is now being studied.



## Major Findings

The goal of this project is a fundamental understanding of the mechanisms by which specific oncogenes transform cells, through the isolation and analysis of cells genetically resistant to transformation. A major effort during the past several years has involved the isolation and analysis of "flat" revertant cells with defects in cellular genes or proteins that are necessary for transformation by selected oncogenes. We have previously isolated two such revertant cell lines with ouabain as a selective agent. These revertants have been used to separate oncogenes that are able to transform a given revertant from oncogenes that cannot. From our previous work, it seems likely that revertants resist transformation because they have defects in an as yet unknown cell-encoded pathway necessary for transformation by the *ras* gene and by other oncogenes that use the same pathway--this interpretation implies that the oncogenes behave differently because they are functionally different. If this is true, then isolation of additional revertants with defects in different pathways could be used to define major functional groups of oncogenes. Since these two initial revertants (C-11 and F-2) exhibited identical patterns of susceptibility and resistance when tested in retransformation assays with a panel of oncogenes, we attempted to isolate additional oncogenes with different behavior patterns. Since the properties of each revertant cell line might reflect the type of selective procedure used in its isolation, we developed a new selective procedure using *cis*-hydroxyproline (CHP). With CHP as the selective agent, two similar revertants were isolated. The new CHP-derived revertants exhibit a very different resistance pattern than the original ouabain-derived revertants.

If we combine the data from the ouabain-resistant revertants with that from the new CHP-derived revertants, the following pattern emerges:

RESISTANCE TO CHP-DERIVED  
REVERTANTS

		<u>yes</u>		<u>no</u>	
RESISTANCE TO OUABAIN-DERIVED	<u>yes</u>	Ha-ras	Ki-ras	<i>src</i>	<i>abl</i>
		N-ras	<i>bas</i>	<i>fgf</i>	<i>fes</i>
REVERTANTS					
	<u>no</u>	<i>raf</i>	<i>fos</i>	<i>fms</i>	<i>sis</i>
		<i>mos</i>			

Thus, these two sets of revertants can be used to define four groups of oncogenes, hopefully according to the cellular pathways with which they interact. Therefore, despite an almost unlimited number of oncogenes and growth factors, there appear to be a relatively limited number of pathways involved in cell transformation. Note that, except for *fms* and *sis*, each of which may act through its own unique receptor pathway (the *sis* protein p28 is identical to the B chain of PDGF, and *fms* is related to the CSF-1 receptor), all of the tyrosine kinase-encoding oncogenes fall into the same group. This indicates that they all use the same pathway to transform cells and that tyrosine kinase activity is a

critical function in this process. The group labeled "1" contains only the *ras*-related genes. To account for the ability of *ras*-derived resistant cells to suppress transformation by other oncogenes and to explain some additional data of Smith *et al.*, using micro-injection of antibodies against p21<sup>ras</sup>, it can be assumed that p21<sup>ras</sup> interacts with the cellular pathway involved in transformation at a point distal to the action of many other oncogene products.

In an extension of our studies with the ouabain revertants resistant to transformation by the *ras* oncogene, Dr. Makoto Noda, a former Visiting Fellow in this section, has recently isolated sequences from a cDNA library described made by Chen and Okoyama from normal human fibroblasts that have the capability of suppressing transformation by *ras* in mouse cells. When sequenced, this activity turns out to be associated with a gene, K-rev 1, that is distantly related to the *ras* gene itself and makes its own version of p21<sup>ras</sup>. Thus, the human cells may be resistant to transformation by *ras* because they have a p21-related product that successfully competes with authentic p21 for the immediate cell-encoded "target" used by the *ras* gene product to transform cells. Other explanations for the activity of K-rev 1 are possible.

Although K-rev 1 does not seem to involve a change in the cellular transformation pathway as we had hoped, this result clearly indicates that we can identify and isolate cDNA sequences capable of reversing the transformed phenotype using the methods we have developed. Because of Dr. Noda's work, which has been confirmed by Drs. Chardin and Tavitian in Paris, we will change our original strategy of transfecting genomic DNA from revertants and will now concentrate on efforts to generate cDNA libraries with revertant activity. We also have isolated several new resistant cell lines that should be useful in generating subtraction cDNA libraries enriched for genes that can induce the revertant phenotype. These include (1) revertant cDNA-NIH/3T3 RNA or transformed NIH/3T3 DNA; (2) revertant cDNA-RNA extracted from re-revertants; and (3) revertant cDNA-RNA from azacytidine-treated revertant cells (revertants treated with azacytidine become stably transformed by an as yet unknown mechanism, perhaps involving a loss of expression of the target gene cell-encoded "target" gene).

A second project deals with an assay for the detection of additional human oncogenes and for the retroviral oncogene *myc*. Although many oncogenes have been isolated to date from human tumor-derived DNA extracts by transfection into cells such as NIH/3T3, fully 80% of all human tumors do not contain any detectable oncogenic activity. There are several models that may account for these negative results, including the possibility that morphological transformation in the majority of cells either *in vitro* or *in vivo* may involve more than a single measurable dominant event. If this is true for a significant number of human tumors, perhaps the search for dominant oncogenes may detect only a fraction of the transforming activity in DNA from human tumor cells. To approach this problem, we chose to examine the standard conditions for transfection of genomic DNA extracts from human tumors for any procedure that might suppress the appearance of transformed foci by hitherto unknown oncogenes.

We established a serum-free assay system that would allow NIH/3T3 cells to survive in culture without passage for periods of up to 6 weeks without the appearance of "background" or spontaneous foci that occur regularly in conventional assay systems. Using "PC-1", a low protein, semi-defined medium from Ventrex Corporation, we observed focus formation in 30% of human tumor DNA extracts that had previously scored as negative in the standard transfection

assay. The cells from each focus were each found to contain *alu* sequences, and the sequences responsible for focus formation are currently under investigation.

To investigate the possibility that an inhibitor of transformation was present in standard transfection assays carried out with serum, we tried to find a known oncogene with known DNA and protein structures to use as a model. It was important to find such an oncogene to enable us to investigate the mechanism of inhibition with well-known probes. If there was such an inhibitor in serum, it might be present in human serum, and this finding might be of importance in controlling analogous human tumors in patients that had somehow lost inhibitor activity. The *myc* oncogene appeared to be a suitable candidate. *myc* is frequently involved in animal and human disease, often following its overexpression and/or translocation. Despite its involvement in tumor formation, *myc* does not readily transform 3T3-like fibroblast lines.

We find that under our serum-free conditions, *myc*, inserted into a retroviral vector with *neo*, is able to transform a specific clone of NIH/3T3 cells, but does not transform other rodent cell lines, including some additional clones of NIH/3T3. The transformation efficiency is very high when the number of *myc*-transformed colonies is compared to the number of *neo*-resistant colonies in the same experiment. When *myc*-infected cells are returned to medium containing serum, they lose the transformed phenotype but still continue to synthesize *myc*-specific mRNA in roughly the same amounts as they do when propagated in serum-free medium. It is possible that the inhibition of *myc*-induced transformation by serum results from effects on a cell-encoded "target" of *myc* rather than on *myc* itself. We are also trying to identify the serum-associated inhibitor of *myc*.

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- Talbot, N., Hankey, P., Jiang, B., Yanagihara, K., Ciardiello, F., Bassin, R.H., and Benade, L.: A system for deriving revertants of oncogene-transformed human cells. *Cytogen. Cell Genet.* 1988; 48:112-116.
- Ciardiello, F., Sanfilippo, B., Yanagihara, K., Kim, N., Tortora, G., Bassin, R.H., Kidwell, W.R., and Salomon, D.S.: Differential growth sensitivity to 4-*cis*-hydroxy-L-proline of transformed rodent cell lines. *Cancer Res.* 1988; 48:2483-2491.
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- Ciardiello, F., Kim, N., Valverius, E.M., Colucci-d'Amato, G., Bassin, R.H., and Salomon, D.S.: Differential growth factor expression in transformed NIH-3T3 cells. *J. Cell. Biochem.*, in press.
- Bassin, R.H., Benade, L., and Noda, M.: Mechanism of transformation as indicated by revertants derived from oncogene-transformed cells. In: Klein, G. (Ed.): *Genes That Counteract Neoplastic Transformation*, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09003-07 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

The Role of TGF $\alpha$  in the Etiology and Progression of Breast Cancer

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

David S. Salomon	Supv., Res. Biochemist	LTIB, DCBD, NCI
Fortunato Ciardiello	Visiting Fellow	LTIB, DCBD, NCI
Eva Valverius	Guest Researcher	Lombardi Cancer Ctr., Georgetown Univ.

COOPERATING UNITS (if any) Dr. Robert H. Bassin, Chief, Biochem. Onc. Sec., LTIB, DCBD, NCI; Dr. Robert Callahan, Chief, Oncogen. Sec., LTIB, DCBD, NCI; Dr. Marc Lippman, Dir., Lombardi Cancer Ctr., Georgetown University, Washington, DC; Dr. Rik Derynck, Dept. Molecular Biology, Genentech, Inc., San Francisco, CA.

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Biochemistry of Oncogenes Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.7

## PROFESSIONAL:

2.7

## OTHER:

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.)

Transforming growth factor alpha (TGF $\alpha$ ) has been circumstantially implicated in the autocrine growth of a number of different rodent and human tumor cells *in vitro*. However, its distribution and role in the etiology and/or progression of rodent and human breast cancer are relatively unknown. The present studies have demonstrated that biologically active and immunoreactive TGF $\alpha$  can be detected in hormone (estrogen)-dependent, DMBA- or NMU-induced rat mammary adenocarcinomas and that these tumors possess a specific 4.8-kb TGF $\alpha$  mRNA. These tumors also express elevated levels of c-Ha-ras protein (DMBA tumors) or possess a point-mutated c-Ha-ras gene (NMU tumors). Ovariectomy results in tumor regression and is preceded by a specific decrease in TGF $\alpha$  mRNA and protein. Transformation of nontransformed mouse mammary epithelial cell lines with a point-mutated c-Ha-ras protooncogene results in the loss in mitogenic responsiveness of these cells to exogenous epidermal growth factor (EGF) and a reduction in the number of unoccupied EGF receptors on these cells due in part to an enhanced production and secretion of TGF $\alpha$ . Several human breast cancer cell lines also secrete TGF $\alpha$  and possess TGF $\alpha$  mRNA. In the estrogen-responsive breast cancer cell lines, estrogen can induce a 3- to 5-fold increase in TGF $\alpha$  mRNA and protein. Treatment of these cells with a polyclonal anti-TGF $\alpha$  antibody or with a monoclonal anti-EGF receptor antibody can inhibit the growth of these cells *in vitro*. Elevated levels of TGF $\alpha$  protein or expression of TGF $\alpha$  mRNA can be detected in 50-60% of primary human breast tumors. No evidence was found for any gross amplifications and/or rearrangements of the TGF $\alpha$  gene in these tumors. Additionally, overexpression of a human TGF $\alpha$  gene in a cloned immortalized population of mouse mammary epithelial cells can lead to the malignant transformation of these cells *in vitro* and *in vivo*. Collectively, these results suggest that TGF $\alpha$  can function as an autocrine growth factor for a subset of rodent and human breast tumors, that estrogens or activated protooncogenes such as ras can enhance the expression of TGF $\alpha$ , and that enhanced production of TGF $\alpha$  can lead to the transformation of mammary epithelial cells that have a sufficient complement of functional EGF receptors.

### Major Findings

We have demonstrated that primary dimethylbenz( $\alpha$ )anthracene (DMBA)- or nitrosomethylurea (NMU)-induced rat mammary adenocarcinomas produce biologically active TGF $\alpha$  that can compete in an EGF radioreceptor assay for binding to the EGF receptor and elaborate an equivalent amount of immunoreactive TGF $\alpha$ . In addition, a specific 4.8-kb TGF $\alpha$  mRNA species could be detected in these tumors after Northern blot hybridization with a labeled nick-translated human TGF $\alpha$  cDNA insert. The primary, carcinogen-induced rat mammary tumors are estrogen dependent for their growth *in vivo* and are estrogen responsive *in vitro*. Ovariectomy results in a rapid and specific reduction in the levels of TGF $\alpha$  mRNA and bioactive TGF $\alpha$  protein in the tumors within 6-12 hr post-ovariectomy. Furthermore, estrogen treatment of primary cultures of DMBA or NMU rat mammary epithelial cells results in a 3- to 4-fold increase in the levels of TGF $\alpha$  mRNA. These results demonstrate that the levels of TGF $\alpha$  in a subset of rat mammary tumors may be regulated by estrogens *in vivo* and *in vitro*. The DMBA-induced rat mammary tumors express elevated levels of c-Ha-ras mRNA and p21<sup>ras</sup> protein, whereas the NMU-induced tumors possess a point-mutated c-Ha-ras gene. In immortalized rodent fibroblasts transformed by *ras*, there is generally an enhanced level of production of TGF $\alpha$  that can be detected in these cells. To determine whether a similar phenomenon may occur in mammary epithelial cells, we have examined the levels of TGF $\alpha$  production in two immortalized mouse mammary epithelial cell lines transformed with a point-mutated, human c-Ha-ras protooncogene. We feel that this may be particularly relevant to human breast cancer, because approximately 60-70% of primary human breast tumors express elevated levels of c-Ha-ras mRNA or p21<sup>ras</sup> protein. Mouse mammary NMuMg cells or cloned NOG-8 cells were transfected with the point-mutated c-Ha-ras gene in plasmids containing either an SV40 promoter (NMuMg) or the glucocorticoid-inducible mouse mammary tumor virus (MMTV) promoter (NOG-8). After transformation of the NMuMg cells with the SV40 plasmid in which overexpression of the point-mutated c-Ha-ras gene is constitutive, there was a 3- to 4-fold increase in the levels of TGF $\alpha$  that could be detected in the conditioned medium (CM). Likewise, after dexamethasone treatment of NOG-8 cells that are carrying the MMTV-inducible construction, there was a rapid increase in the levels of a 1.4-kb specific c-Ha-ras mRNA and p21<sup>ras</sup> protein within 3-12 hr. During the same interval, there was a concomitant increase in the levels of TGF $\alpha$  mRNA that was followed by a subsequent elevation in the amounts of TGF $\alpha$  in the CM. Normally, NMuMg or NOG-8 cells are mitogenically responsive to exogenous EGF in monolayer cultures and will grow in soft agar to a limited degree in response to exogenous EGF or TGF $\alpha$ . However, after *ras* transformation, the cells become refractory to these exogenously added growth factors. This may be due in part to the enhanced levels of endogenous TGF $\alpha$  that are secreted and occupy cell surface EGF receptors on these cells. This may occur because the number of unoccupied EGF receptors on these cells is reduced by 80-90%, suggesting that there has been a chronic receptor down-regulation induced by overexpression of *ras*. There is also an increase in TGF $\beta$  and insulin-like growth factor type I expression following *ras* transformation in the NOG-8 cells, suggesting that a spectrum of complementing growth factors are induced in mammary epithelial cells by this activated protooncogene and that part of the enhanced anchorage-dependent and -independent growth may be mediated through these growth factors.

TGF $\alpha$  is not entirely restricted to malignant rodent mammary epithelial cells. Biologically active and immunoreactive TGF $\alpha$  (range 0.5 to 50 ng/10<sup>8</sup> cells/48 hr) has been detected in the CM obtained from the human breast cancer cell lines MCF-7, ZR-75-1, T47-D, and MDA-MB-231. In the estrogen-responsive and estrogen

receptor (ER)-positive and progesterone receptor (PgR)-positive breast cancer cell lines MCF-7, ZR-75-1, and T47-D,  $17-\beta$  estradiol ( $10^{-8}$  M) produced a 3- to 5-fold increase in the CM levels of TGF $\alpha$  within 24 hr after treatment of the cells. Preceding this increase in secreted TGF $\alpha$  protein, there was a corresponding elevation in the level of expression of a major 4.5- to 4.8-kb TGF $\alpha$  mRNA species and a minor 1.6-kb transcript that could be detected within 6 hr. Optimal induction occurs between  $10^{-9}$  and  $10^{-8}$  M estrogen. Biosynthetic labeling of MCF-7 or MDA-MB-231 cells with [ $^{35}$ S]cysteine followed by immunoprecipitation of the culture medium with a specific polyclonal rabbit anti-TGF $\alpha$  antibody demonstrated the presence of several high molecular weight TGF $\alpha$  species between 17,000 and 30,000. Treatment of several of these breast cancer cell lines with a neutralizing polyclonal anti-TGF $\alpha$  antibody or with a blocking mouse monoclonal anti-EGF receptor antibody produced a 60-70% inhibition in the anchorage-dependent and -independent growth of these cells, suggesting that this growth is functioning through the EGF receptor in an autocrine fashion to regulate the proliferation of these cells. To ascertain if overexpression of the TGF $\alpha$  gene in a population of nonmalignant, immortalized mammary epithelial cells is sufficient to induce their neoplastic transformation, we co-transfected an SV40-human TGF $\alpha$  cDNA expression vector plasmid and the pSV2neo plasmid into the NOG-8 mouse mammary epithelial cell line. After selection with G418 for several weeks, we isolated a number of G418-resistant clones. Analysis of the CM from a number of these isolates demonstrated that several clones were secreting high levels of TGF $\alpha$  in the range of 200 to 600 ng/ $10^8$  cells/48 hr as compared with 5-10 ng/ $10^8$  cells/48 hr for the parental nontransfected or only the pSV2neo-transfected cells. Those clones that were secreting elevated levels of the growth factor grew aggressively in soft agar and formed undifferentiated, invasive carcinomas when injected into nude mice at a frequency of 80-90% within a period of 2-3 weeks. Additionally, there was an 80-90% reduction in the number of free, unoccupied EGF receptors on these cells compared with the parental NOG-8 cells. Neutralizing mouse monoclonal anti-TGF $\alpha$  antibodies were able to produce a 60-70% inhibition in the soft agar growth of these cells. Collectively, these results demonstrate that overexpression of this growth factor in an immortalized population of mammary epithelial cells that also possesses a sufficient complement of functional EGF receptors is capable of transforming these cells.

We have also initiated a series of studies to ascertain whether TGF $\alpha$  is produced by primary human breast carcinomas and to determine whether there are any genomic alterations in this gene within these tumors. In detergent extracts prepared from normal or benign mammary tissues and from 22 primary infiltrating ductal carcinomas, the immunoreactive TGF $\alpha$  concentrations were found to range from 1.5 to 6 ng/mg cell protein, with approximately 50% of the breast tumors possessing TGF $\alpha$  levels that were  $>2.5$  ng/mg cell protein, the level that was at the upper end for the normal and benign tissues. We also analyzed 54 infiltrating ductal carcinomas for TGF $\alpha$  mRNA expression. Approximately 50-70% of these tumors obtained from two separate clinical sources expressed a detectable 4.8-kb TGF $\alpha$  mRNA species. However, no significant association could be discerned between TGF $\alpha$  mRNA expression and ER and/or PgR status, axillary lymph node involvement, c-Ha-ras expression, and patient relapse due to the recurrence of the primary tumor or distal metastasis. Furthermore, in a total of 79 breast tumors that were analyzed by Southern blot hybridization after digestion of high molecular weight DNA with BamHI, no gross rearrangements or amplifications of the TGF $\alpha$  gene could be detected when compared with the DNA restriction pattern that was obtained from samples of matched peripheral blood lymphocytes from the same breast cancer patients. We have been able to demonstrate the presence of significantly higher levels (approximately 2- to 3-fold) of biologically active and immunoreactive TGF $\alpha$  in the serous effusions (pleural and ascites) from breast

cancer patients compared with the levels found in the effusions from noncancer patients. In addition, there appears to be a significant positive correlation between elevated levels of TGF $\alpha$  in these effusions and the presence of two other tumor-associated antigens, CEA and TAG-72. These data suggest that TGF $\alpha$  levels in effusions may serve as a prognostic marker for breast cancer recurrence.



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Shankar, V., Ciardiello, F., Kim, N., Derynck, R., Liscia, D.S., Merlo, G., Langton, B.C., Sheer, D., Callahan, R., Bassin, R.H., Lippman, M.E., Hynes, N., and Salomon, D.S.: Transformation of normal mouse mammary epithelial cells following transfection with a human transforming growth factor alpha cDNA. *Mol. Carcinogenesis*, 1989, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04829-15 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

The Identification and Characterization of Human Genes Associated with Neoplasia

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

Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI
Giorgio Merlo	Visiting Associate	LTIB, DCBD, NCI
Danielle Liscia	Visiting Associate	LTIB, DCBD, NCI
Craig Cropp	Biotechnology Fellow	LTIB, DCBD, NCI
Kajanubu Fujita	Guest Worker	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. Carleton Garrett, George Washington Univ. Med. School, Washington, DC; Dr. Rosette Lidereau, Centre Rene Huguenin, St. Cloud, France; Dr. Mariani-Costantini, Univ. of Rome, Italy; Dr. Gregory Campbell, Lab. of Statistics and Mathematical Methodology, NIH; (see attached sheet)

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Oncogenetics Section

## INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.4

## PROFESSIONAL:

4.4

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

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

A/B

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

The etiology of human breast cancer is thought to involve a complex interplay of genetic, hormonal, and dietary factors that are superimposed on the physiological status of the host. Attempts to derive a cohesive picture of how these factors participate in the etiology of breast cancer have been confounded by a lack of information on specific mutations associated with the initiation and progression of the disease. We have undertaken a strategy that is aimed at determining, on a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant association of patient history, tumor characteristics, and patient postsurgical prognosis. The *int-2* gene is frequently activated in mouse mammary tumors by the mouse mammary tumor virus (MMTV) via insertional mutagenesis. We previously found that *int-2* is amplified 2- to 15-fold in 14% of primary human breast tumor DNAs (n = 118) and has a significant association (p < 2 × 10<sup>-6</sup>) with a poor prognosis for the patient. We have now defined the amplification unit on chromosome 11q13 and found that 17 of 18 tumors amplified for *int-2* were also amplified for *hst* and *bcl-1*. The *hst* and *int-2* genes are both related to basic fibroblast growth factor. The *bcl-1* locus defines the translocation t(11;14) breakpoint in B-cell chronic lymphocytic leukemias. In a lymph node metastasis of a breast cancer patient, *int-2* but not *hst* was expressed. In 10% of the primary breast tumor DNAs (n = 122) the *c-erbB-2* gene was amplified. The *c-erbB-2* gene is related to the gene encoding the epidermal growth factor receptor. However, in contrast to one published report, we found no significant association with patient postsurgical prognosis or other aspects of patient history and tumor characteristics. In other studies, we detected the expression of MMTV-related human RNA sequences in breast tumor-derived cell lines and have obtained recombinant cDNA clones of these sequences for further analysis.



## Cooperating Units (continued)

Dr. Henry Lynch, Creighton Univ., Omaha, NE; Dr. Fredrick Li, Epidemiology Branch, DCBD, NCI; Dr. Alberto P.M. Cappa, Pathology Service, Ospedale Maggiore S. Giovanni, Turin, Italy; Dr. Bruce Roe, Univ. of Oklahoma, Norman, OK; Dr. Gordon Peters, Imperial Cancer Research Fund, London, England.

### Major Findings

In previous work on frequently occurring mutations in primary human breast tumor DNA, we found the *c-myc* proto-oncogene amplified in 32%, and the *int-2* proto-oncogene amplified in 16% of the tumors, respectively. We have further characterized the *int-2* amplification unit on chromosome 11q13 by testing other closely linked markers. These include the proto-oncogenes *c-sea* and *hst*, the t(11;14) translocation breakpoint *bcl-1*, and the apolipoprotein gene complex APOE-1, as well as the progesterone receptor gene (chromosome 11q21) and the *ets-1* proto-oncogene (chromosome 11q23). In 17 of 18 tumors containing an *int-2* gene amplification only *bcl-1* and *hst* were coamplified. More recently, we have obtained preliminary evidence, by RNA:RNA *in situ* hybridization, suggesting that *int-2* but not *hst* RNA is expressed in a lymph node metastasis from a breast cancer patient.

We have found that the *c-erbB-2* proto-oncogene is amplified in approximately 10% of our primary breast tumor panel. Univariate and multivariate analysis of various clinical parameters, including patient prognosis and overall survival, did not reveal a significant association with *c-erbB-2* amplification. This is in contrast to a recent report by Slamon *et al.* (*Science* 235:177, 1987) claiming that *c-erbB-2* gene amplification has a highly significant association with relapse and overall survival. This apparent conflict may reflect the absence of certain controls in the case of Slamon *et al.* as well as aspects of their statistical methodology.

In previous work, we identified and characterized a family of human endogenous retroviral genomes (designated HERV-II) that share significant homology with MMTV structural genes. Recently, we have identified two breast tumor cell lines (MDA-MB-468 and MDA-MB-231) that express RNA species (5.0 and 2.0 kb) related to the HERV-II genomes. In addition, the MDA-MB-468 cell line also expresses another novel 1.7-kb RNA species that hybridizes only with HERV-II long terminal repeats. Recombinant cDNA libraries from these cell lines have been constructed and are being screened for HERV-II-related clones.

*Publications*

Callahan, R.: Two families of human endogenous retroviral genomes, in eucaryotic transposable elements as mutagenic agents. In *Eucaryotic Transposable Elements as Mutagenic Agents. Banbury Reports*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988, pp. 91-100.

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Ciardiello, F., Kim, N., Hynes, N., Jaggi, R., Redmond, S., Liscia, D.S., Sanfilippo, B., Merlo, G., Callahan, R., Kidwell, W.R., and Salomon, D.S.: Induction of transforming growth factor expression in mouse mammary epithelial cells after transformation with point-mutated c-Hras protooncogene. *Mol. Endocrinol.*, 1988; 2:1202-1216.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05148-10 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Mammary Tumorigenesis in Inbred and Feral Mice

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

Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI
Gilbert Smith	Research Biologist	LTIB, DCBD, NCI
Antonio Marchetti	Visiting Fellow	LTIB, DCBD, NCI
Daniel Gallahan		LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. Francesco Squartini, U. of Pisa, Pisa, Italy; Dr. Bruce Roe, U. of Oklahoma, Norman, OK; Dr. Nigel Spurr, Imperial Cancer Research Fund, Potters Bar, England; Dr. Sandy Smith-Gil, NCI; Dr. Scott Freeman, NIAID; Dr. Jamie Zweibel, Georgetown University, Washington, DC; Dr. Glenn Merlino, NCI

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Oncogenetics Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

1.5

## OTHER:

0.8

## CHECK APPROPRIATE BOX(ES)

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

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The study of experimentally induced mammary tumors has focused primarily on several mouse strains that are infected with mouse mammary tumor virus (MMTV) and have been inbred for a high incidence of mammary tumors. We have expanded this study to include other species of MMTV-infected feral mouse strains that have not been bred for high mammary tumor incidence. MMTV appears to induce mammary tumors by acting as an insertional mutagen that leads to the activation of previously silent cellular genes (*int* genes). We have previously shown that the inbreeding program used to develop the high-incidence mouse strains may also have fixed other cellular mutations that affect the frequency with which *int-1* and *int-2* are activated. The *int-1* and *int-2* genes are much less frequently activated in feral mouse mammary tumors, raising the possibility that other *int* loci are active in these tumors. We described one such locus, designated *int-3*, in CZECHII V<sup>+</sup> feral mice. The organization of *int-3* differs from that of *int-1* or *int-2*. MMTV insertions occur within a 500-bp region of the cellular genome on chromosome 17. Viral insertion at this locus leads to the activation of the expression of two cellular RNA species that correspond to host flanking sequences located 5' and 3' of the viral insertion site, respectively. We are currently determining the nucleotide sequence of recombinant cDNA clones corresponding to each of these RNA species. Mice chronically infected with MMTV frequently contain preneoplastic hyperplastic alveolar nodules (HAN), which can be transplanted as hyperplastic outgrowth (HOG) lines. We have found that in three of four CZECHII HOG lines, *int-1* is occupied by an MMTV genome. This suggests that activation of *int-1* is an early event in MMTV-induced mammary tumorigenesis. Human breast tumors frequently express tumor-associated antigens. One of these, the carcinoembryonic antigen (CEA) is a member of the family of related glycoproteins that are expressed normally in fetal tissue, the placenta, and other adult tissues. We have found that MMTV-induced CZECHII V<sup>+</sup> mammary tumors express a 4.0-kb RNA species that is related to human CEA.



### Major Findings

We have previously found that 18% of CZECHII V<sup>+</sup> mammary tumors (n = 44) contain a MMTV proviral genome integrated at the *int-3* locus. MMTV integration at *int-3* activates the expression of a 6.5- and 2.6-kb RNA species corresponding to cellular sequences located 5' and 3', respectively, of the viral insertion site. In two of the tumors, however, only the 2.6-kb RNA species was detected. In these tumors the MMTV proviral genome at the *int-3* locus was truncated, containing only long terminal repeat related sequences. At the present time, we are determining whether or not the cellular sequences located 5' of the viral insertion site are expressed in these tumors. We have obtained recombinant cDNA clones of portions of the 6.5- and 2.6-kb *int-3* RNA species and are now determining their nucleotide sequences.

In *M. spretus* (MS) and CZECHII V<sup>+</sup> mammary tumors, the *int-1* and *int-2* genes are activated at a significantly lower frequency (*int-1* 0% and 24% respectively, *int-2* 2% and 6% respectively) than in mammary tumors of high-incidence mouse strains. This may reflect differences in the tropism of the feral mouse MMTV strains for subpopulation of mammary epithelium that are sensitive to the activation of other *int* loci. To test this possibility, we have developed new mouse strains by foster nursing BALB/c and CZECHII V<sup>-</sup> newborns on MS mice and BALB/c newborns on CZECHII V<sup>+</sup> mice. We have immunological evidence of virus transmission in each of these foster-nursed mouse strains and have begun to collect mammary tumors to determine the frequency with which the known *int* genes are activated. If the frequency of *int* gene activation is the same as in the virus donor strain, it would strongly suggest that the frequency of *int* gene activation is, in part, a function of the particular virus strain.

Mammary epithelium of mice chronically infected with MMTV frequently contain preneoplastic lesions termed hyperplastic alveolar nodules (HANs). HANs are composed of immortalized cells capable of repopulating mammary fat pads that have been cleared of epithelium. HANs transplanted in this manner are referred to as hyperplastic outgrowth (HOG) lines. We have found that the *int-1* locus in three of four CZECHII HOG lines is occupied by an acquired MMTV proviral genome. This suggests that activation of *int-1* represents an early event in mammary tumorigenesis which contributes to the immortalization of mammary epithelium.

CEA is frequently expressed in primary human breast tumors as well as other neoplasias. It is a member of a family of related high molecular weight glycoproteins, some of which are expressed in fetal tissue, the placenta, and certain normal adult tissues. Using a recombinant cDNA probe representing the 3' portion of the human CEA gene, we have detected a related 4.0-kb species of RNA in MMTV-induced CZECHII V<sup>+</sup> mammary tumors. We have obtained a recombinant cDNA clone representing a portion of the mouse CEA-related RNA and are presently determining its nucleotide sequence.

*Publications*

Gallahan, D., Kozak, C., and Callahan, R.: A new common integration region (*int-3*) for mouse mammary tumor virus on mouse chromosome 17. *J. Virol.*, 1987; *61*:218-220.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 09023-03 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

Cloning of Anti-Tumor Antigen Immunoglobulin Genes

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

Syed Kashmiri	Expert	LTIB, DCBD, NCI
Paula Hutzell	Biologist	LTIB, DCBD, NCI
Benjamin Calvo	Staff Fellow	LTIB, DCBD, NCI
Anna Maria Masci	Visiting Fellow	LTIB, DCBD, NCI
Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBD, NCI
Jeffrey Schlom	Chief	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. Mark Bodmer, Celltech, Inc., England

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Oncogenetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.2

## PROFESSIONAL:

4.2

## OTHER:

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 main objective of this research project is to genetically engineer immunoglobulin genes to generate useful reagents for *in vivo* localization and therapy of human tumors. Several hybridoma cell lines have been developed in this laboratory that produce monoclonal antibodies (MABs) that selectively react with tumor-associated antigens. These include the carcinoembryonic antigen and a tumor-associated glycoprotein, TAG-72, which is present in a variety of carcinomas. The MABs are currently being used in a number of diagnostic and therapeutic trials on breast, colon, and ovarian cancers. In clinical tests, one such MAB, B72.3, which was raised against a membrane-enriched extract of a human metastatic breast carcinoma and recognizes TAG-72, has shown promise for being developed into a diagnostic and therapeutic agent. The usefulness of mouse MABs for *in vivo* therapy and diagnosis of human tumors, however, is limited because of their immunogenicity in patients. This problem can be reduced by replacing the constant region of the mouse antibody with the constant region of the human antibody, using recombinant DNA techniques. To that end, we have cloned the rearranged heavy and lightchain variable regions as well as the cDNA copies of the heavy and light chain gene messages of the MAB, B72.3. Chimeric heavy and light chain immunoglobulin genes have been constructed. They were inserted separately into appropriate expression vectors. Stable chimeric antibody-producing cell lines were developed by introducing these constructs into both fibroblast and myeloma cell lines. Chimeric antibody produced either from cDNA clones or clones of the rearranged genes retained specificity and binding properties of the parental antibody.

### Major Findings

We have prepared mouse-human chimeric B72.3 antibodies from cells transfected with expression constructs of chimeric cDNA or genomic B72.3 clones.

A cDNA library was constructed, in  $\lambda$ gt10 vector, using cDNA synthesized from mRNA isolated from the hybridoma cell line, B72.3. The library was screened with radiolabeled probes specific to the CH1 domain of mouse  $\gamma$ -1 and Ck sequences. Two *EcoRI* fragments, one of 980 bp and the other of approximately 1700 bp, containing intact coding sequences for leader, variable, and constant regions of light ( $\kappa$ ) and heavy chains, respectively, were detected and cloned. The *EcoRI* cDNA inserts were subcloned in M13 vectors, and the variable regions of heavy and  $\kappa$  chain clones were sequenced by the chain termination procedure of Sanger. Chimeric mouse-human constructs were generated by replacing the constant region of B72.3 heavy and  $\kappa$  chain cDNA clones with the rearranged human  $\gamma$ -4 and human Ck region, respectively. In order to make a chimeric mouse-human heavy chain construct, a *HindIII* site was created by site-directed mutagenesis at the start of the CH1 exon of a genomic clone of human  $\gamma$ -4. The B72.3 variable region of heavy chain cDNA clone, which has a *BglIII* site near its 3' end, was fused to the human  $\gamma$ -4 region using a *BglIII-HindIII* synthetic linker. Construction of mouse-human light chain clone was straight forward because both mouse and human Ck regions have a site for *MboII* 18 bp downstream from the junction of variable and constant domains. The chimeric genes were inserted separately in an expression vector that contains a strong promoter-enhancer transcriptional control element from human cytomegalovirus. The expression constructs were introduced either singly or in heavy/light chain gene pairs in COS-1 cells that allowed high transient expression of the chimeric antibody molecules. An antigen-based ELISA assay showed that the chimeric antibody molecules retained the specific binding characteristics of the parent B72.3 antibody. SDS-PAGE analysis of the supernatant from transfected COS-1 cells labeled with [<sup>35</sup>S]-methionine showed that transiently expressed antibodies were correctly synthesized and assembled in tetrameric form. To develop a stable cell line producing chimeric antibody, an expression construct of the mouse-human chimeric B72.3 light chain gene was introduced into a vector carrying the neo resistant gene and was electroporated into CHO-K1 cells. Transfectants were selected in medium supplemented with G418 and screened for light chain producers by an ELISA assay. Expression constructs of chimeric B72.3 heavy chain gene carrying *gpt* as a selectable marker were then electroporated into CHO-K1 cells producing light chain. Mycophenolic acid-resistant colonies were assayed for antibody production by antigen-binding assay. Stable transfectants producing 0.1-40  $\mu$ g/ml of chimeric antibody were identified. Chimeric antibody produced by the transfectant maintained the tissue binding and idiotypic specificity of the native B72.3 antibody.

As a first step toward cloning the rearranged gene, the unique restriction fragments carrying functionally rearranged genes were to be identified. To that end, Southern blot analysis of genomic DNA of NS1, Balb/c, and hybridoma cell line B72.3 was undertaken. To identify the light chain, a genomic  $J_{\kappa}$  fragment was used as a probe. The rearranged B72.3 light chain gene was identified on a unique 18-kb *BamHI* fragment of B72.3 genomic DNA. A partially enriched genomic library was prepared from the 18-kb fraction of *BamHI*-digested B72.3 DNA in EMBL3 vector. The library was screened with both the J region probe and a variable region probe derived from B72.3 light chain cDNA clone. Southern blot analysis of *EcoRI*-digested B72.3 genomic DNA with  $J_{\kappa}$  probe showed 3-kb and 6.5-kb fragments in B72.3 DNA that were not present in NS1 and Balb/c genomic DNA fragments. A partially enriched genomic library was constructed from the 3-kb fraction of *EcoRI*-digested B72.3 genomic DNA in  $\lambda$  Zap vector. The library was



screened with  $J_H$  and variable region probe derived from the cDNA clone. Clones of rearranged light and heavy chain genes were isolated, their inserts were characterized by restriction endonuclease mapping, and they were subcloned in M13 vector for sequencing. Sequence analysis of light and heavy chain gene clones showed their sequences to be identical to the B72.3 light and heavy chain cDNA clones. Construction of mouse-human chimeric light chain gene was performed by inserting a 5.6 kb *Hind*III fragment containing  $\kappa$  VJ region of B72.3 into the *Hind*III site of pACYC 184/HNEK vector, which contains sequences encoding the human C $\kappa$  region and the neo resistant gene as a selectable marker. Similarly, the mouse-human chimeric heavy chain gene was constructed by inserting an approximately 3 kb *Eco*RI fragment containing rearranged VDJ sequences of B72.3 into *Eco*RI sites of PSV2 *gpt*/Hu IgG<sub>1</sub> vector that includes constant region of the genomic human IgG<sub>1</sub>. SP2/0 cells were electroporated with expression construct carrying chimeric light chain genes. Transfectants were selected for G418 resistance, and expression of light chain gene was analyzed by Western blot analysis. The chimeric H2L2-producing cell line was developed by electroporating the chimeric heavy chain expression construct into SP2/0 cells producing light chain. Mycophenolic acid-resistant transfectants were assayed for H2L2 production by TAG-72 antigen-based radioimmunoassay. Several H2L2-producing colonies were picked that produced 5.0-10.0  $\mu$ g/ml of chimeric 72.3 Mab.

*Publications*

Whittle, N., Adair, J., Lloyd, C., Jenkins, L., Devine, J., Schlom, J., Raubitschek, A., Colcher, D., and Bodmer, M.: Expression in COS cells of a mouse-human chimaeric B72.3 antibody. *Protein Eng.* 1987; 6:499-505.

Colcher, D., Milenic, D., Roselli, M., Raubitschek, A., Yarranton, G., King, D., Adair, J., Whittle, N., Bodmer, M., and Schlom, J.: Characterization and biodistribution of recombinant and recombinant/chimeric constructs of monoclonal antibody B72.3. *Cancer Res.* 1989; in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09027-01 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Biological Effect(s) of Oncogenetic Elements on Mammary Cell Growth and Senescence

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

Gilbert H. Smith

Research Biologist

LTIB, DCBD, NCI

Robert Callahan

Chief, Oncogenetics Section

LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Robert Bassin, LTIB, DCBD, NCI; David Salomon, LTIB, DCBD, NCI; Dennis Roop, Baylor College of Medicine, Houston, TX; Daniel Medina, Baylor College of Medicine, Houston, TX; Jaime Zweibel, Georgetown University School of Medicine, Washington, DC; L.J.T. Young, University of California, Davis, CA

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Oncogenetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

none

## CHECK APPROPRIATE BOX(ES)

- (a) Human 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 number of genetic elements have been implicated in the process of malignant transformation in the mammary glands of experimental animals and humans. The mouse mammary gland provides an excellent experimental model within which to elucidate the function of these putative mammary oncogenes during the growth, development, and differentiation of mammary tissue *in vivo*. Any portion of the adult mouse mammary gland at any age and throughout all stages of differentiation can repopulate the gland-free mammary fat pad of syngeneic mice upon transplantation. Similar results are obtained when dissociated glandular epithelium is injected into the fat pad or alternatively grown in culture under prescribed conditions prior to reintroduction into the mammary fat pad. We have initiated experiments designed to evaluate the feasibility of introducing functional genetic elements into mouse mammary cells with recombinant retroviral expression vectors antecedent to the reintroduction of these cells into a gland-free fat pad. Paramount to the success of such a project is the maintenance of the "repopulation potential" of the mammary epithelial cells. Our studies and those of others indicate that the number of cells capable of repopulation of the fat pad is small initially and the ability to repopulate is gradually lost through mitotic events. We have morphologically identified a potential candidate for these "mammagenic" cells *in situ* and in explant cultures. These cells seem to differentially express certain cytokeratins during growth and expansion of the mammary gland *in vivo*, suggesting that specific keratin expression could serve as a marker for these "stem" cells. In addition, primary mammary cell cultures are being evaluated for the efficiency of retroviral gene transfer, the level of recombinant trans-gene expression in the infected cultures, and the stability of recombinant retroviral gene expression in "mammagenic cell populations" subsequent to repopulation of the gland-free mammary fat pads. We have developed a number of partially transformed preneoplastic mammary outgrowth lines which are carried serially *in vivo* by transplantation into gland-free mammary fat pads. Cells from these lines have been grown *in vitro* and are capable of repopulating mammary fat pads subsequently. These cells are being tested for retroviral gene transfer studies.

### Major Findings

Sixteen hyperplastic mammary outgrowth lines have been established from Czech II V<sup>+</sup> as well as Czech II V<sup>-</sup> mice that had been foster-nursed on GR and C3H high-incidence inbred mouse strains. Five of these serially transplantable preneoplastic mammary populations have begun to produce mammary tumors in a stochastic manner. The cellular DNA from 4 of these 5 lines has been analyzed for MMTV DNA insertions which result in the rearrangement of the known *int* genes, *int-1*, *int-2*, and *int-3*. Three hyperplastic lines were found to possess a rearrangement at *int-1*; the tumors arising within these lines possess the same *int-1* rearrangement. These observations suggest that these lines are clonal. Analysis of DNA from the original outgrowth confirmed this conclusion. The fourth hyperplastic line that was analyzed did not have MMTV rearrangements near *int-1*, *int-2*, or *int-3*; but did contain 2 newly acquired MMTV genomes. The tumors arising from this line also contain these same integrations, again suggesting that this hyperplasia developed clonally. Analysis of the regions flanking these MMTV insertions may reveal additional *int* genes important in the early stages of mammary tumorigenesis.

Transplantation experiments were conducted to test the "repopulation potential" of the normal mammary gland in various stages in its development and differentiation. All portions of the gland were capable of repopulating gland-free mammary fat pads within 12 weeks of implantation. The estimated minimum number of cells required for this activity was approximately  $2.3 \times 10^3$ , but these fragments contained the growing points (end buds) of actively developing mammary epithelium in pubescent female mice. From mature virgins the smallest number of cells that was capable of producing a repopulated gland was a fragment of the tertiary duct or about  $7.2 \times 10^3$  epithelial cells. In contrast approximately  $3.3 \times 10^4$  epithelial cells were contained within the fragments of lactating mammary gland implanted within the gland-free pad. Interestingly, these latter fragments were less efficient at repopulation although possessing larger numbers of epithelial cells. In agreement with these transplantation studies we were able to detect morphologically distinct cells present among the epithelium which persisted throughout all stages of development and differentiation. These cells were more prevalent in the fragments of growing end buds and tertiary ducts of 6 week old mice. These fragments were able to repopulate the cleared fat pad most efficiently. In explant culture, these "stem" cells underwent mitosis within 4 hrs even in the absence of DNA synthesis, giving rise to groups of morphologically distinct, pale-staining cells. In the presence of lactogenic hormones, these daughter cells differentiated to a secretory phenotype. We believe these cells represent dormant mammary epithelial-specific precursor cells. We are currently evaluating keratin gene expression in the developing and differentiated mammary epithelium in an effort to determine if these gene products could serve as cellular markers for these putative mammary epithelial stem cells.

Frozen sections of normal mouse mammary gland were stained with antibodies raised against the synthetic peptides unique to the following epidermal keratins (K1, K10, K5, K14, K6, K16, K18, K13). These studies were carried out in collaboration with Dr. Dennis Roop (Baylor). Two pairs of keratin genes were expressed differentially in mouse mammary epithelium, K6 and K16 and K5 and K14. The polypeptides encoded by these genes combine within the cellular cytoplasm to form keratin-specific intermediate filaments. K5 and K14 were expressed in basally located epithelia which apparently corresponded to the myoepithelial cells. K6 and K16 were found associated with a subset of luminal epithelial cells which possessed the same distribution in developing, differentiating, and



functioning mammary epithelium as the putative mammary stem cells we described earlier. The numbers of these K6-positive cells increased during periods of epithelial growth and expansion in a manner similar to the pale-staining morphologically distinct cells. Expression of K6 by these cells was confirmed directly by immunoperoxidase staining of semi-thin sections of plastic-embedded mammary explants. We are currently examining preneoplastic and neoplastic mammary tissues for the expression of these keratins.

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Smith, G.H. and Medina, D.: A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. *J. Cell Sci.* 1988; 89:173-183.

Liscia, D.S., Doherty, P.J., and Smith, G.H.: Localization of  $\alpha$ -casein gene transcription in sections of epoxy-resin-embedded mammary tissues by *in situ* hybridization. *J. Histochem. Cytochem.* 1988; 36:1503-1510.

Ali, I.U., Campbell, G., Merlo, G.R., Smith, G.H., Callahan, R., and Lidereau, R.: Multiple genetic alterations in human breast cancer and their possible prognostic significance. In *Cold Spring Harbor Symposia on Cancer Cells*, Vol. 7. *Molecular Diagnostics of Human Cancer*. 1989, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08281-07 LTIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Role of cAMP in Growth Control and Differentiation: Gene Regulation

## 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
H. Yokozaki	Visiting Fellow	LTIB, DCBD, NCI
G. Tortora	Visiting Fellow	LTIB, DCBD, NCI
T. Clair	Chemist	LTIB, DCBD, NCI
S. Ally	Guest Worker	LTIB, DCBD, NCI

## COOPERATING UNITS (if any)

Dr. M. Medniekes, U. of Chicago, Wyler Children's Hospital, Chicago, IL; Dr. Tore Jahnsen, Institute of Pathology, Rikshospitalet, Oslo, Norway; Dr. H. Fan, U. of California, Irvine, CA; Dr. C. Wychowski, Institut Pasteur, Paris, France; Dr. D. Ogreid, U. of Bergen, Bergen, Norway

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Cellular Biochemistry Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

2.0

## OTHER:

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 prokaryotes, cAMP is known to regulate the transcription of specific genes by a well-defined mechanism. cAMP binds to the prokaryotic cAMP binding protein CRP (cAMP receptor protein)/CAP (catabolite gene activator protein), and this complex interacts with specific DNA sequences at the 5' ends of targeted genes, modulating the ability of RNA polymerase to bind to the promoters. In mammalian cells, gene expression is altered in response to stimulation by hormones. Stimuli whose action is mediated through adenylate cyclase and cAMP-dependent protein kinase influence the transcriptional efficiency of several genes through the binding of specific factors to distinct *cis*-acting DNA sequences, CRE (cAMP responsive element), located in the 5' noncoding (promoter) region. Recently, a region containing the CRE has been shown to bind specific nuclear proteins from various tissues. Recent evidence suggests that transcriptional regulation of eukaryotic genes by cAMP requires a cAMP-dependent protein kinase. In this study, we examined the role of cAMP-dependent protein kinase in gene transcription. Since cAMP-dependent protein kinase is exclusively present in the cytoplasm, any nuclear function of the protein kinase must be accompanied by its translocation to the nucleus. It was found that the *ras* gene suppression, growth inhibition, differentiation (leukemic cells), and phenotypic reversion of Ha-MuSV-transformed NIH/3T3 cells by site-selective cAMP analogs correlated with the nuclear translocation of the RII cAMP receptor protein, the regulatory subunit of protein kinase type II, which was detected within 10 min after the analog treatment. Thus, the nuclear translocation of the RII cAMP receptor protein is an early event in the cAMP regulation of growth and differentiation. The goal of this study is to provide direct evidence of interaction between the cAMP consensus sequences and cAMP receptor protein (RII) and/or a phosphoprotein substrate of protein kinase.

### Major Findings

Type I and type II cAMP-dependent protein kinases are distinguished by their regulatory subunits (RI and RII, respectively). Four different regulatory subunits ( $RI_\alpha$  [previously designated RI],  $RI_\beta$ ,  $RII_\alpha$  [ $RII_{54}$ ] and  $RII_\beta$  [ $RII_{51}$ ]) have been identified at the gene/mRNA level. Two different catalytic subunits ( $C_\alpha$  and  $C_\beta$ ) have also been identified; however, preferential coexpression of either one of these catalytic subunits with either the type I or type II protein kinase regulatory subunit has not been found. The changing levels of protein kinase isozymes, therefore, can be measured using the photoaffinity ligand 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP, which binds covalently to their regulatory subunits, the cAMP receptor proteins. The untreated breast (MDA-MB-231 and MCF-7) and colon (LS-174T) cancer cells and HL-60 leukemia cells contained a major cAMP receptor protein with a  $M_r$  of 48,000, the  $RI_\alpha$  cAMP receptor protein. When the cells were treated for 3 days with 8-Cl-cAMP, the cAMP receptor protein of 52,000  $M_r$ ,  $RII_\beta$ , increased appreciably with a concomitant decrease of the  $RI_\alpha$  protein. The concentration, subcellular localization, and transcriptional control of the expression of these cAMP receptor proteins have been examined during treatment of LS-174T human colon cancer cells with 8-Cl-cAMP. Two species of RII with an apparent  $M_r$  of 52,000 ( $RII_\beta$ ) and 56,000 ( $RII_\alpha$ ) and a single species of RI with a  $M_r$  of 48,000 ( $RI_\alpha$ ) were identified in the cancer cells.  $RI_\alpha$  and both forms of RII were covalently labeled with 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP, and two different anti-RII antibodies that exclusively recognize either  $RII_\beta$  or  $RII_\alpha$  resolved two forms of the RII receptors. 8-Cl-cAMP treatment induced a decrease of  $RI_\alpha$ , an increase of both  $RII_\beta$  and  $RII_\alpha$  in the cytosols of cancer cells, and rapid translocation (within 10 min) of  $RII_\beta$  from the cytosol to nucleus. Nuclear run-off assay demonstrated that 8-Cl-cAMP caused transcriptional activation of the  $RII_\beta$  receptor gene and inactivation of the  $RI_\alpha$  receptor gene. The transcriptional control of these cAMP receptor genes, however, did not appear to be the initial event in 8-Cl-cAMP-induced growth inhibition. Within 10 min after 8-Cl-cAMP treatment, both  $RI_\alpha$  and  $RII_\beta$  receptor levels increased 2-fold even though the transcriptional changes of these genes could not be detected yet. Thus, 8-Cl-cAMP initially enhanced, at least transiently, the stability of both  $RI_\alpha$  and  $RII_\beta$  receptors and simultaneously induced a rapid nuclear translocation of  $RII_\beta$  but not  $RI_\alpha$  receptor protein. In K562 chronic myelogenous leukemic cells, Northern blot analyses demonstrated that 8-Cl-cAMP treatment induced an increase in the  $RII_\beta$  mRNA level along with a decrease in the  $RI_\alpha$  mRNA level but did not affect the mRNA level of the catalytic subunit. 8-Cl-cAMP treatment also brought about a rapid decrease in c-myc mRNA level. These changes occurred within a few hours of analog treatment, indicating that the changes are early events rather than the consequences of growth inhibition and differentiation. The growth inhibition induced by 8-Cl-cAMP of a human lung carcinoma, LX-1, in athymic mice also correlated with a selective modulation of different forms of cAMP receptor proteins.  $RI_\alpha$  and  $RII_\beta$  levels changed in an inverse manner during 8-Cl-cAMP treatment. Northern blotting analyses demonstrated that the changing levels of  $RI_\alpha$  and  $RII_\beta$  receptors paralleled the changing levels of their mRNAs. Also, only  $RII_\beta$ , not  $RI_\alpha$ , accumulated in the nucleus in regressing tumors after 8-Cl-cAMP treatment. 8-Cl-cAMP treatment also caused a sharp decrease in both N-ras and c-myc mRNA levels in the tumors.

These results suggest that the fundamental basis for the antineoplastic activity of 8-Cl-cAMP resides in the restoration of normal gene regulation in neoplasms in which cAMP receptor proteins may play a role. To further explore the role of cAMP receptor proteins in the gene regulation of cancer cells, we are currently conducting experiments that employ antisense strategy and gene transfer technology.



*Publications*

Cho-Chung, Y.S., Clair, T., Tagliaferri, P., Ally, S., Katsaros, D., Tortora, G., Neckers, L., Avery, T., Crabtree, G., and Robins, R.K.: Site-selective cAMP analogs as a new biological tool in growth control, differentiation, and proto-oncogene regulation--basic science review. *Cancer Invest.* (In press).

Tortora, G., Ciardiello, F., Ally, S., Clair, T., Salomon, D.S., and Cho-Chung, Y.S.: Site-selective 8-chloroadenosine 3',5'-cyclic monophosphate inhibits transformation and transforming growth factor  $\alpha$  production in Ki-ras-transformed rat fibroblasts. *FEBS Lett.* 1989;2:363-367.

Ally, S., Clair, T., Katsaros, D., Tortora, G., Finch, R.A., Avery, T.L., and Cho-Chung, Y.S.: Inhibition of growth and modulation of gene expression in human lung carcinoma in athymic mice by site-selective 8-Cl-cAMP. *Cancer Res.* (In press).

Ally, S., Tortora, G., Clair, T., Grieco, D., Merlo, G., Katsaros, D., Ogreid, D., Døskeland, S.O., Jahnsen, T., and Cho-Chung, Y.S.: Selective modulation of protein kinase isozymes by site-selective 8-Cl-cAMP provides a biological means for control of human colon cancer cell growth. *Proc. Natl. Acad. Sci. USA* 1988;85:6319-6322.

Tortora, G., Clair, T., Katsaros, D., Ally, S., Colamonici, O., Neckers, L.M., Tagliaferri, P., Jahnsen, T., Robins, R.K., and Cho-Chung, Y.S.: Induction of megakaryocytic differentiation and modulation of protein kinase gene expression by site-selective cAMP analogs in K-562 leukemic cells. *Proc. Natl. Acad. Sci. USA* (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 CB 05216-18 LTIB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Site-Selective cAMP Analogs as Antineoplastics and Chemopreventives</b>		
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
H. Yokozaki	Visiting Fellow	LTIB, DCBD, NCI
G. Tortora	Visiting Fellow	LTIB, DCBD, NCI
S. Ally	Guest Worker	LTIB, DCBD, NCI
COOPERATING UNITS (if any) Dr. B. Jastorff, University Bremen, FRG; Dr. W.R. Miller, U. of Edinburgh, Scotland; Dr. S.O. Døskeland, U. of Bergen, Norway; Dr. P. Tagliaferri, U. of Naples Medical School II, Naples, Italy; Dr. T. Avery, Nucleic Acid Research Institute, Costa Mesa, CA		
LAB/BRANCH Laboratory of Tumor Immunology and Biology		
SECTION Cellular Biochemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Cyclic AMP (cAMP) in mammalian cells functions by binding to cAMP receptor protein, the regulatory subunit of cAMP-dependent protein kinase. The cAMP receptor protein has two different cAMP binding sites, and cAMP analogs that selectively bind to either one of the two binding sites are known as Site 1-selective (C-2 and C-8 analogs) and Site 2-selective (C-6 analogs), respectively. Such site-selective cAMP analogs at micromolar concentrations exerted potent growth inhibition, with no sign of toxic effects, in a spectrum of human cancer cell lines, including those that are resistant to previously tested cAMP analogs, leukemia lines, v-ras-H oncogene-transformed NIH/3T3 cells, and v-ras<sup>K1</sup> oncogene- or TGFA-transformed normal rat kidney (NRK) cells; the combination of a C-8 with a C-6 analog showed synergistic effects. The growth inhibition was not due to a block in a specific phase of cell cycle but paralleled selective modulation of type I versus type II protein kinase isozymes, suppression of cellular proto-oncogene expression, inhibition of TGFA production, phenotypic change, and differentiation (leukemic cells). It is suggested that the site-selective cAMP analogs, substituting for endogenous cAMP and binding to protein kinase, suppress the cancer cell growth not via directly inhibiting cell division but rather by promoting cell differentiation. Site-selective cAMP analogs thus provide new biological tools for investigating cell proliferation and differentiation and also for the improved management of human cancer.</p> <p>8-C1-cAMP is now in preclinical phase I studies at the National Cancer Institute. A U.S. patent for the cAMP analogs, entitled "Derivatives of cyclic AMP as treatment for cancer," was applied for on May 25, 1988.</p>		

### Major Findings

New site-selective cAMP analogs demonstrated the growth inhibition with many-fold greater potency than previously studied analogs in a spectrum of 20 human cancer cell lines. Of 24 site-selective analogs tested, the majority produced growth inhibition (30-80%) with no sign of toxicity in all 20 cancer cell lines at micromolar concentrations. The growth inhibition was not due to a block in cell cycle progression but paralleled a change in cell morphology, an augmentation of the RII cAMP receptor protein, and a decrease of p21ras protein. Since the adenosine counterpart of the analog produced cell death and G<sub>1</sub> synchronization without affecting the RII and p21ras protein levels, it is likely that an adenosine metabolite is not involved in the analog effect. The effect of site-selective cAMP analogs on the growth of rat mammary tumors and human tumor xenografts, MX-1 and MDA-MB-231 mammary carcinomas, LOVO colon carcinoma, and LX-1 lung carcinoma, in athymic mice was also tested. These *in vivo* models were used to determine the efficacy as well as toxicity of cAMP analogs. Studies demonstrated that the site-selective analogs that are most potent in the growth inhibition of the cancer cell lines in culture are also most effective in producing tumor regression *in vivo*.

The effect of site-selective cAMP analogs on the expression of differentiation markers was determined in leukemic cells. Treatment of HL 60 cells for 3 days with 8-Cl-cAMP at concentrations where 90% cell viability is present, induced a marked increase in the expression of monocyte-specific surface antigens (MO<sub>2</sub> and OKM<sub>1</sub>) and a decrease in markers related to the immature progenitor cells (My7 and My9). Disappearance of cellular terminal deoxynucleotidyl transferase (TdT) has been considered as a differentiation marker for human T-lymphocytic leukemia. Treatment of MOLT-4 (acute T lymphocytic) leukemia cells with 8-Cl-cAMP (10 μM) caused a time-dependent decrease in TdT activity; by Day 4, the activity decreased to 10% of the untreated control levels. Moreover, treatment for 4 days with 8-Cl-cAMP in combination with N<sup>6</sup>-benzyl-cAMP (20 μM) brought about almost complete loss (over 95%) of TdT activity. In the K-562 chronic myelogenous leukemia cell line, site-selective cAMP analogs induced megakaryocytic differentiation. Treatment for 3 days with 8-Cl-cAMP (30 μM) induced a marked increase in the fraction of cells positive for the glycoprotein II<sub>b</sub>-III<sub>a</sub> antigen, a specific megakaryocytic marker expressed in the differentiation of this lineage. N<sup>6</sup>-benzyl-cAMP treatment alone did not induce an appreciable increase of this antigen expression, but when N<sup>6</sup>-benzyl-cAMP was added with 8-Cl-cAMP, the majority of cells expressed this antigen.

Transforming growth factor alpha (TGFα) is a potent mitogen for fibroblasts and epithelial cells. 8-Cl-cAMP (50 μM) exerted potent inhibition of both the monolayer and soft agar growth of NRK fibroblasts that had been transformed with the v-ras<sup>K1</sup> oncogene or treated with TGFα. The growth inhibition was dose dependent and reversible and was accompanied by reversion of the transformed phenotype, suppression of TGFα production, and a decrease in p21ras protein levels. These effects were linked to the cAMP analog's selective modulation of the type I versus type II protein kinase regulatory subunits present in these cells. These data support the direct role of 8-Cl-cAMP in the control of malignancy induced by certain growth factors and/or cellular oncogenes.

These data show that site-selective cAMP analogs provide a biological tool for control of malignant growth.

## Publications

- Cho-Chung, Y.S.: Site-selective cAMP analogs in the arrest of cancer cell growth. In: *Developments in Cancer Chemotherapy Vol II*. R. Glazer (Ed.), CRC Press, Boca Raton, FL, 1989, pp. 78-93.
- Tagliaferri, P., Katsaros, D., Clair, T., Ally, S., Tortora, G., Neckers, L., Rubalcava, B., Parandoosh, Z., Chang, Y.A., Revankar, G.R., Crabtree, G.W., Robins, R.K., and Cho-Chung, Y.S.: Synergistic inhibition of growth of breast and colon human cancer cell lines by site-selective cAMP analogs. *Cancer Res.* 1988;48:1642-1650.
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Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1988 to September 30, 1989

During the past year there have been several administrative changes. Dr. Borsos retired from his position as Chief of the Laboratory of Immunobiology and became a Scientist Emeritus. Dr. Zbar was appointed Chief of the laboratory.

In the Cellular Immunity Section, studies continued on the genetic basis of human kidney and lung carcinomas. Analysis of tumors from patients with von Hippel-Lindau disease provided strong evidence that there is a tumor suppressor gene located on chromosome 3p. Multiple renal cell carcinomas in individual patients with von Hippel-Lindau disease showed loss of the same chromosome 3p alleles, demonstrating that the same chromosome was deleted in each tumor. Central nervous system hemangioblastomas and a pheochromocytoma also showed 3p allele loss. Analysis of haplotypes indicated that the loss of chromosome 3p alleles was from the chromosome bearing the balancing, wild-type allele of the VHL gene. These results are consistent with the concept that the VHL gene is a recessive oncogene.

To begin to define the location of the VHL gene we have assembled a panel of families with von Hippel-Lindau by contacting ophthalmologists, urologists, neurosurgeons and medical geneticists around the United States and Canada. To date we have identified 22 families containing some 105 living, affected individuals. Blood samples have been collected from 419 individuals. There are about 220 individuals at risk of developing the illness. Individuals at risk are being examined in the Clinical Center under the protocol "Von Hippel-Lindau disease: early diagnosis and identification of genetic markers closely linked to the disease gene".

Linkage of VHL was found to cRAFl, a gene located at 3p24-3p25. The LOD score was 5.80 at a recombination distance of 0.12. Multipoint linkage analysis is in progress. Current data suggest that the VHL gene is located distal to cRAFl in the telomeric region of chromosome 3p. There was no evidence for genetic heterogeneity. Familial pheochromocytoma was linked to cRAFl.

Three renal oncocytomas were examined for loss of alleles on chromosome 3p. In contrast to sporadic and hereditary renal cell carcinomas, renal oncocytomas did not show a loss of alleles at loci on chromosome 3p. This result supports the view that renal oncocytoma is a distinct form of renal neoplasia.

Efforts to clone the von Hippel-Lindau disease gene are continuing. To date we have isolated from a chromosome 3 library more than 4000 phage clones that contain single copy human DNA sequences. The inserts have been isolated using low melting point agarose gels. These inserts are being used in a large screening program designed to: a) assign regional location; b) identify probes that contain genes; c) identify probes that detect differential expression of message in tumor cells compared to their normal

counterparts. So far, the screening program has yielded more than 400 chromosome 3p probes and among them, 100 probes that detect DNA sequences conserved between different mammalian species. Of 24 conserved probes, 15 detected transcripts in renal cell carcinoma cells and small cell lung carcinoma cells and 12 detected useful RFLPs.

In the Immunopathology Section, major progress was made on three different biologically active proteins discovered in this laboratory. The first was called monocyte-derived neutrophil chemotactic factor (MDNCF) in our previous report; it has been re-named neutrophil attractant/activation protein-1 (NAP-1) and is referred to by some laboratories as interleukin-8 (IL-8). NAP-1 attracts human neutrophils, but not monocytes, in contrast to many well-studied attractants that do not have this leukocyte specificity. Thus, it could contribute to acute inflammatory responses characterized by neutrophil infiltration. In studies on interaction with other types of leukocytes, NAP-1 did not attract eosinophils and attracted about 10% of a normal human basophil population. A study with Dr. Martha White showed that NAP-1 caused release of histamine from human basophils. In collaboration with Dr. Dennis van Epps, it was found that NAP-1 attracted human cultured T-lymphocytes. To determine the biological significance of the above in vitro effects, quantitative evaluation has begun on the action of NAP-1 injected intradermally into human skin. Unlike C5a, which causes a global response that includes a wheal-and-flare as well as mast cell degranulation, NAP-1 has no observable effect on skin mast cells, and the response appears to be confined to neutrophil infiltration. It is possible that NAP-1 is an important mediator in diseases characterized by neutrophil-rich inflammatory lesions. As a first step in the study of NAP-1 in lung disease, a collaboration has been initiated with Dr. John Rankin at Yale to determine if normal lung macrophages obtained by bronchoalveolar lavage make NAP-1. These cells were put into culture and stimulated with bacterial endotoxin. Culture fluids were analyzed for chemotactic activity and NAP-1 ELISA reactivity before and after passage down anti-NAP-1 columns. The experiments showed that stimulated lung macrophages secreted NAP-1, and that the culture fluid contained attractants in addition to NAP-1. The presence of NAP-1 was confirmed by isolation and partial sequencing of the purified product.

Additional insight into the chemistry of NAP-1 was obtained as a result of a new purification procedure that reduced the number of manipulations. NAP-1 was purified on an immunoaffinity column, after which HPLC-CM chromatography yielded pure NAP-1 in 4 distinct peaks. N-terminal amino acid sequence analysis, done by Elizabeth Robinson in the laboratory of Dr. Ettore Appella, showed that in addition to the 72-residue protein originally found, there were two other forms that had 5 and 7 additional residues at the N-terminus. It was shown that incubation of crude culture fluid at 37°C resulted in conversion of the longer forms of NAP-1 to the 72-residue form, by proteolysis at a R-S bond. Further proteolysis did not occur (despite other R-S bonds in the protein), which suggests that this phlogistic molecule may persist at sites of release and contribute to chronicity of inflammation in pathologic foci.

A new chemotactic cytokine, monocyte chemoattractant protein-1 (MCP-1), was purified to homogeneity in the Immunopathology Section during the past year; and in collaboration with Dr. Teodoro Appella and colleagues it was sequenced and cloned. In contrast to NAP-1, this protein attracts human monocytes and does not interact with human neutrophils. A key to success in purification of this protein was the discovery that a human glioma cell line secretes large amounts, even in the absence of serum, so that only a 150-fold purification was required to obtain the pure product. MCP-1 was purified from glioma culture fluid sequential Orange-A-Sepharose chromatography, carboxymethyl (CM)-HPLC, and reverse phase HPLC. It is a basic protein, with a molecular mass of 3400. It appears as two distinct peaks on CM-HPLC and as two bands with slightly different mobility on SDS-PAGE. The indistinguishable amino acid composition of these peaks suggests that the differences are due to post-translational modification. The identical pair of proteins was also isolated from PHA-stimulated human blood mononuclear cells, which suggests that MCP-1 is the attractant that mediates accumulation of monocytes in classical delayed hypersensitivity reactions.

Studies on the molecular biology of MCP-1 provided new insights about this protein. Northern blot analysis of 5 glioma cell lines showed a correlation between the intensity of the message and the amount of MCP-1 secreted. Several other types of human tumor cell lines revealed no MCP-1 message. Stimulation of blood mononuclear cells caused appearance of MCP-1 message in response to PHA and IL-1 beta, but not to IL-2, TNF-alpha, or IFN-gamma. Under conditions of high stringency, MCP-1 cDNA hybridized with genomic DNA from chimpanzee and baboon, but not rat or mouse. Mitogenic stimuli of several different cell types cause increases in mRNAs that have sequence similarity to the coding sequence of MCP-1. The greatest similarity (68% matching of the nucleotides of the coding region) is with a sequence called JE, which increases when mouse fibroblasts are stimulated by PDGF. This suggests that MCP-1 is the human homologue of the JE gene product. JE mRNA is increased not only by PDGF in fibroblasts, but by endotoxin stimulation of mouse macrophages and by EGF stimulation of rat endothelial cells. The generation of JE/MCP-1 by mitogenic stimuli emphasizes that both proliferation (for wound healing) and cell recruitment (for host defense) are part of the response to tissue injury.

Macrophage stimulating protein (MSP), a protein found in mammalian blood plasma, makes mouse macrophages responsive to chemoattractants and causes macrophage pseudopod formation. MSP has now been purified to near homogeneity from 20 liters of human plasma, with a yield of approximately 500 micrograms. The essential steps are application of plasma to a monoclonal anti-MSP antibody column, acid elution of MSP (5000-fold purification), and two ion exchange HPLC steps (an additional 100-fold purification). On SDS-PAGE, pure MSP is seen as a major band with a molecular mass of about 90,000 daltons. Under reducing conditions, there are two bands, with masses of 60,000 and 30,000. Thus, MSP comprises two polypeptide chains, linked by disulfide bonds. After reduction and alkylation, the two chains were separated by gel filtration under denaturing conditions. After endopeptidase digestion of the chains and separation of the resulting fragments, partial amino acid sequences of several fragments were established. Comparison of these sequence data with sequences in the data bank for existing proteins shows that MSP is a newly described protein.



Office of the Chief. In collaboration with the Mainz group we continued elucidation of the mechanism of the activation of C1, the first component of complement. C1 is made up of five subcomponents, 2 C1s, 2 C1r and 1 C1q molecules. These subcomponents dissociate as a function of ionic strength and concentration and  $Ca^{++}$  are necessary for association. In the fluid phase reassociation of the dissociated C1 is highly efficient and can result in the reconstitution of the original intact molecule. We have compared the association of the subcomponents in the fluid phase with reassociation when C1q is bound to an antibody-antigen complex. We discovered that reassociation of bound C1q with the other subcomponents is inefficient: less than 0.1% of the macromolecule is in the associated state. This finding is important for one of the functions of the C1 inhibitor molecule is the disassembly of C1 into its subcomponents; if disassembly occurs after C1 was bound to antibody-antigen complexes, reassociation is effectively blocked. Since the biological function of bound C1q is very different from bound C1, the consequences of inefficient reassembly have important implications for transport, removal and digestion of antibody-antigen complexes.

Several years ago it was reported that mouse oncornavirus were inactivated by human but not by mouse serum. It was shown that the virus bound and activated human C1 while mouse C1 was bound but not activated. The activated C1 activated the classical C cascade and the result was virus neutralization. No antibody was involved in the reaction. We studied the inactivation of HIV-1 virus by fresh normal sera of several mammals and discovered that numerous murine and feline sera were capable of inactivating HIV-1, that the inactivating activity was heat labile, required  $Ca^{++}$  and there was no antibody involved. Human and non-human primate sera lacked this activity. The observation is consistent with the hypothesis that non-primate serum complement directly inactivates HIV-1 and that lack of inactivation by primate sera may be due to lack of activating interaction of C1 in these sera with the virus. This line of study is now pursued vigorously.



Major findings: In collaboration with the Mainz group we continued elucidation of the mechanism of the activation of C1, the first component of complement. C1 is made up of five subcomponents, 2 C1s, 2 C1r and 1 C1q molecules. These subcomponents dissociate as a function of ionic strength and concentration and Ca<sup>++</sup> are necessary for association. In the fluid phase reassociation of the dissociated C1 is highly efficient and can result in the reconstitution of the original intact molecule. We have compared the association of the subcomponents in the fluid phase with reassociation when C1q is bound to an antibody-antigen complex. We discovered that reassociation of bound C1q with the other subcomponents is inefficient: less than 0.1% of the macromolecule is in the associated state. This finding is important for one of the functions of the C1 inhibitor molecule is the disassembly of C1 into its subcomponents; if disassembly occurs after C1 was bound to antibody-antigen complexes, reassociation is effectively blocked. Since the biological function of bound C1q is very different from bound C1, the consequences of inefficient reassembly have important implications for transport, removal and digestion of antibody-antigen complexes.

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#### Publications

Hosoi S, Borsos T. Further evidence for dilution dependent dissociation of C1q in human serum. *Complement and Inflammation* 1989, in press.

Borsos T. Histories and mysteries about C1. *Behring Institute Mitteilungen* Nr. 84, 1989.

Borsos T. Immune complex activation of the classical complement pathway. *Behring Institute Mitteilungen* Nr. 84, 1989.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08575-16 LIB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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: T. Yoshimura Visiting Fellow

## COOPERATING UNITS (if any)

Ettore Appella Laboratory of Cell Biology, DCBD

## LAB/BRANCH

Laboratory of Immunobiology

## SECTION

Immunopathology Section

## INSTITUTE AND LOCATION

NCI, NIH, ECRF, Frederick, MD 21701

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

2.5

## OTHER:

1.0

## 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.)

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: our recently discovered monocyte-derived neutrophil chemotactic factor (MDNCF) has been renamed neutrophil attractant/activation protein-1 (NAP-1) and is referred to by some laboratories as interleukin-8. We have made studies on the leukocyte specificity of NAP-1, previously shown to attract human neutrophils but not monocytes. NAP-1 did not attract eosinophils, but did attract about 10% of a normal human basophil population. In collaboration with Dr. Martha White, it was shown that NAP-1 caused histamine release from human basophils. And in a study with Dr. Dennis van Epps, it was found that NAP-1 attracted cultured human T-lymphocytes. We have begun quantitative evaluation of the effects of NAP-1 injected intradermally into human skin. NAP-1 caused neutrophil infiltration, without degranulation of mast cells. To study the role of NAP-1 in generation of neutrophil-rich exudates in human disease, a quantitative ELISA for NAP-1 has been developed. The ELISA has been used in a collaboration with Dr. John Rankin at Yale to establish that LPS-stimulated human bronchoalveolar lavage macrophages secrete NAP-1, and that the macrophage culture fluid contains other neutrophil attractants in addition to NAP-1. Detection of NAP-1 in these fluids by ELISA was confirmed by isolation and partial sequencing of the purified product.

A new purification procedure for NAP-1 was developed. NAP-1 was purified on an immunoaffinity column, after which HPLC-CM chromatography yielded pure NAP-1 in 4 well-separated peaks. N-terminal amino acid sequence analysis showed that in addition to the 72-residue protein originally found, there were two other forms, with 5 and 7 additional residues at the N-terminus. The longer forms were converted by proteolysis at R-S bonds to the 72-residue form.

Monocyte chemoattractant protein-1 (MCP-1), a new chemotactic cytokine, was purified by us during the past year. In collaboration with Dr. Ettore Appella and colleagues, it was sequenced and cloned. This protein attracts monocytes, but not neutrophils. It was purified from both glioma culture fluids and PHA-stimulated human blood mononuclear cells. MCP-1 is thought to be the attractant that mediates accumulation of monocytes in delayed hypersensitivity reactions. Although Northern blots showed that several glioma cell lines made MCP-1 message, a number of other human tumor cell lines showed no MCP-1 message. Blood mononuclear cells expressed MCP-1 message in response to PHA and IL-1 beta, but not to IL-2, TNF-alpha or IFN-gamma. Stimulation of several different cell types causes increases in mRNA's that have sequence similarity to the coding sequence of MCP-1. The coding region of the gene expressed when mouse fibroblasts are stimulated by platelet-derived growth factor (PDGF) has a 68% nucleotide match with that of MCP-1, which suggests that MCP-1 is the human homologue of the JE gene product. The generation of JE/MCP-1 by mitogenic stimuli emphasizes that both proliferation (for wound healing) and cell recruitment (for host defense) are part of the response to tissue injury.

During the past year, the Section has purified Macrophage Stimulating Protein (MSP) to near homogeneity. MSP, a protein found by us in mammalian blood plasma, makes mouse peritoneal macrophages responsive to chemoattractants and also stimulates phagocytic and motile activity. Approximately 500 micrograms of MSP have been purified from 20 liters of

human plasma by sequential immunoaffinity column and CM-HPLC chromatography. MSP has a molecular mass by SDS-PAGE of approximately 90 kd. Under reducing conditions, two bands with masses of about 60 and 30 kd can be detected. After endopeptidase digestion of the separated chains, partial amino acid sequences of several fragments were determined. The sequence data show that MSP is a newly described protein.

#### Publications.

Matsushima K, Morishita K, Yoshimura T, Lavu S, Kobayashi Y, Lew W, Appella E, Kung SF, Leonard EJ, Oppenheim JJ. Molecular cloning of cDNA for a human monocyte derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin-1 and tumor necrosis factor. *J Exp. Med.* 1988;167:1883-1893.

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Kuratsu J, Leonard EJ, Yoshimura T. Production and characterization of human glioma cell derived monocyte chemotactic factor. *JNCI* 1989;81:347-351.

Yoshimura T, Robinson EA, Tanaka S, Appella E, Leonard EJ. Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *J. Immunol.* 1989;142:1956-1962.

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Major findings:

The gene for von Hippel-Lindau disease was found to be linked to cRAF1, a gene located at 3p24-25. The gene for pheochromocytoma was also found to be linked to cRAF1. There was no evidence for genetic heterogeneity.

Tumors (renal cell carcinomas, hemangioblastomas and a pheochromocytoma) from patients with von Hippel-Lindau disease showed loss of alleles at loci on chromosome 3p. Multiple renal cell carcinomas in individual patients with von Hippel-Lindau disease showed loss of the same chromosome 3p alleles, demonstrating that the same chromosome 3p was deleted in each tumor. Analysis of haplotypes indicated that the loss of chromosome 3p alleles was from the chromosome bearing the wild-type allele of the von Hippel-Lindau disease gene.

Isolation of the von Hippel-Lindau disease gene

So far 400 chromosome 3p probes have been isolated. One hundred of these probes detect DNA sequences conserved between different mammalian species.

Renal Oncocytoma

In contrast to sporadic and hereditary renal cell carcinomas, renal oncocytomas did not show a loss of alleles at loci on chromosome 3p. This result supports the view that renal oncocytoma is a distinct form of renal neoplasia.

Adrenal carcinoma

Loss of alleles on chromosomes 11p, 13q and 17p was observed in primary tumors and metastases but not in adrenocortical adenomas or hyperplastic lesions of the adrenal cortex. One patient with adrenocortical carcinoma had a somatic mutation in the HRAS1 gene in the normal adrenal gland.

Publications:

Zbar B. Chromosomal deletions in lung cancer and renal cancer. In: Important Advances in Oncology. Edited by V. DeVita, S. Hellman, S. Rosenberg, Lippincott, 1989, pp 41-60.

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## LABORATORY OF MATHEMATICAL BIOLOGY

### SUMMARY

October 1, 1988 through September 30, 1989

Research in the Laboratory of Mathematical Biology (LTB) covers a broad range of theoretical and experimental studies of biological systems. These studies include molecular modelling, theoretical molecular calculations, membrane structure and function, immunology, pharmacokinetics, and physiological modelling studies. Application of the basic understanding of these biological systems, serving as models for aspects of the cancer and other process, is accomplished through the use of advanced computing. Close collaborations provide valuable feedback and knowledge transfer between research domains. The Laboratory often develops computational and experimental methodology that is utilized by researchers through the biomedical community at large. Many of the theoretical studies have only been possible through the use of supercomputing facilities at the Advanced Scientific Computing Laboratory, FCRF.

#### Office of the Chief

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

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

New analytical tools for studies of proteins and nucleic acids have been developed and implemented (Barber, Le, Maizel, Nussinov, Owens). Numerical methods aid in the prediction of secondary structures, splice sites, promoters, and recombination sites in nucleic acids. Graphic representations reveal homology, and reverse complementarity. These programs were developed and have been installed on a variety of computer systems at the Advanced Scientific Computing Laboratory. Structures up to 2000 bases in size have been predicted.



Methods to assess the significance of predictions have used Monte Carlo simulations, evolutionary comparisons and biochemical data. New sequences were compared with computerized databases to detect relationships with known proteins.

RNA secondary structure methods have been refined to include alternate energy parameters, extended Monte Carlo simulations, and comparative studies to establish firmly the uncommon structural features in subregions of a number of sequences of HIV and other retroviruses (Le, Nussinov, Currey, Shapiro, Maizel). These predicted structural features have been correlated with biological features, leading to deeper understanding of the replication and expression processes in this group of viruses. In HIV-1 and related viruses, predicted stable features have been correlated with sites of *tat*-regulation elements, *rev* responsive elements and sites of translational frame-shift. Conserved secondary structure is predicted to be absent in regions of hypervariability in the envelope gene m-RNA's. Use of the supercomputer has allowed development of a lookup procedure for predicting stability of random sequences, which accelerates surveys nearly 100-fold. Monte Carlo techniques are being developed that yield greater than 80% correct prediction of t-RNA structures, for more than 100 examined sequences.

Techniques are being developed and are leading to sensible three-dimensional models for perforin and apo-lipoproteins. Biochemical data will permit testing of these predicted models. This work is the beginning of what must become a more routine part of gene sequence analysis (Peterson). Preliminary sequence/structure analyses have also been performed on oncogenes, DNA binding regions and reverse-transcriptases (Barber).

Studies on the structure-function relationship of beta(1-4) galactosyltransferase (GT) and its modifier protein, alpha-lactalbumin continued this year (Qasba, Masibay and Boeggeman). The cDNA sequence of bovine GT, constructed from a partial cDNA clone and a genomic fragment, and engineered in an Okayama-Berg vector, upon transfection of COS-7 cells, codes for an enzymatically active GT. The expressed protein is modulated by alpha-lactalbumin to change the acceptor specificity to glucose, to synthesize lactose. The expressed GT protein is fully functional and the binding sites for UDP-galactose, N-acetylglucosamine, glucose, and alpha-lactalbumin are all intact and operative. The GT cDNA sequences have been further engineered to delineate the regions involved in 1) producing either soluble or membrane anchored forms of protein, 2) binding of UDP-galactose, N-acetylglucosamine, glucose and alpha-lactalbumin. Studies are being carried out to characterize the cell-surface GT, the mechanism of synthesis of surface and Golgi transferase, their genes, and the role of cell-surface GT in cell growth.

Molecular Structure. In the laboratory we are studying biological macromolecules and their properties, including studies on peptides, proteins, DNA and RNA. This includes physical chemistry of processes such as folding, binding and changes of conformation.

One principal difficulty in achieving the correct folded conformation of a protein is the overwhelmingly large number of possible conformations. Restricting the space in which the conformations are generated provides a large reduction in the number of feasible folded forms. We have been developing schemes that limit the conformations generated simply by restricting them to be

within a small volume (Jernigan and Covell). It has been possible to enumerate all of the possible folded topologies of several small proteins and evaluate them with simple residue-residue interactions. In a way similar to folding small proteins, studies have begun on tertiary folding of RNA. The same procedure of using regular lattices to divide and simplify conformations has proven useful to investigate the binding of small peptides to larger proteins (Covell, Margalit and Jernigan).

Molecular modeling has been proceeding in four areas: membrane proteins (Guy, Raghunathan, Peitsch), small peptides (Guy, Jiang, Jernigan, and Covell), DNA double helix (Jernigan, Sarai, Mazur, Nussinov, Raghunathan, Shapiro, and Owens), and DNA-protein interactions (Takeda and Sarai). For the membrane proteins this model construction proceeds by combining experimental data with calculations of preferred locations and orientations of helices with respect to membrane boundaries, helix-helix packing, formation of charge pairs and disulfide bonds. Conformational models have been developed for the antibiotic magainin, pardaxin,  $\delta$  lysin, and PGLa; these have assisted in understanding how they can lyse cells and form channels. Small peptide 2D NOE NMR data indicating close atoms have been combined with molecular calculations to yield models for the neuropeptide Substance P and tachykinins. DNA double helices exhibit some dependence on the base sequence; these details are being studied by investigating the sequence dependence of the DNA helix flexibility. Methods to calculate the induction of bends of specific shapes and curvatures are being developed. One question that is unresolved is whether or not the details of these conformations play a functional role in gene regulation. For the DNA-protein interactions in operator-repressor interactions, the modelling proceeds by evaluating the strengths of various classes of interactions.

Image Processing. Two areas of research are pursued in the Image Processing Section (Lipkin): development of analytic methods to analyze two dimensional gels (Lemkin), and nucleic acid secondary structure studies (Shapiro). In the first area, development of the GELLAB-II software system was continued, with concentration on increasing the resolution, gel image scanning, quantitation accuracy, capabilities to annotate results and integration of a user graphics windowing interface. A constant goal has been to simplify the user's role in performing the exploratory data analysis of a composite 2D gel database. A major part of the effort has been in integrating the UNIX based GELLAB-II software with UNIX workstation based X-windows interactive graphics for portability.

GELLAB has been used to find a secreted axonal protein AXONIN-I (Lemkin, Sonderegger, U. Zurich). New studies include cadmium toxicity of epithelial cells (Lemkin, Sonderegger) and one on long term capillary cerebral epithelial cell lines to aid in understanding the blood-brain barrier (Lemkin, Sonderegger, Amberger and Bauer, Salzberg). A continuing project is to analyze sets of lymphocyte gels and long term cell lines from human hematological malignancies to identify potential marker proteins for possible microsequencing (Lemkin, Lester, U. Tenn.). Also, GELLAB-II is being used to help assess stability of long term cell lines in multiple passages (Lemkin, Alley).

The RNA secondary structure analysis system has expanded to include more functionality for analyzing RNA conformations from various perspectives (Shapiro, Maizel). This has involved the development of new algorithms to explore secondary structure motifs and RNA tertiary structure, and has been made

available as a computer workbench to allow a researcher interactively to pursue areas of interest concerning RNA structure. Facilities are included to explore multiple structures as well as individual RNA structures. This system permits queries of relationships of RNA secondary structures and can involve the various software/hardware objects available at the FCRF. The L11 operon of *E. coli* has been studied with respect to regulation effects of the L1 protein. Also, the structural effects of the translation efficiency of the lamB gene of *E. coli* were studied.

Studies on the use of concepts of information theory in understanding molecular sequence patterns have advanced through analysis of experimental results showing that the patterns at T7 phage promoters, unlike several other prokaryotic recognition sites, have more sequence information than necessary for transcription (Schneider). Work is under way to provide a theoretical description of molecular machinery such as enzymes, ribosomes, etc., that can set bounds on theoretical capacity and hence aid in design of molecular devices.

Simulation, Analysis and Modelling of Physiological Systems. Development continued on the simulation, analysis, and modeling (SAAM/CONSAM) computer programs (Zech, Grief, and Boston) that permit increased numbers of compartments, components, adjustable parameters and data points. Version 30 of SAAM and CONSAM was developed on the VAX and is available under the VMS operating system. A major effort has been applied to the development of a version of SAAM30/CONSAM30 for personal computers that use the Intel 80386 central processor. Advanced interactive graphics capabilities similar to those available on the VAX or the SUN are under development. Dr. Zech served as Chairman on an international committee to contribute advice regarding the direction of kinetic analysis for a resource in kinetic analysis. The SAAM group organized and participated in workshops on the topics of SAAM/CONSAM in kinetic analysis. A special workshop was organized to discuss the kinetics of lipid and lipoproteins, centered around the use of SAAM and COSAM.

The SAAM project also carried out collaborative research efforts, involving a large number of national and international investigators, in the analysis of data in the fields of lipid and lipoprotein metabolism, the testing of hypotheses concerning human epinephrine and norepinephrine metabolism, the quantitative description of the whole body metabolism and pharmacokinetics of cancer preventive selenium compounds, and the quantitative description of the metabolically significant vitamin A dynamics underlying homeostatic mechanisms that function to regulate the general physiological functions of growth and differentiation, reproduction, and vision and their relationships in cancer prevention. The SAAM project also carried on collaborations with a large number of national and international investigators in the chemical, pharmacological and behavioral aspects of marijuana and "designer" drugs. A general pharmacokinetic compartmental model for universal dosing regimens was developed to facilitate optimization of individual dosage in the clinical setting. This model was demonstrated in the optimization of a theophylline regimen as well as the estimate of pharmacokinetic parameters.

### Theoretical Immunology



The overall aim is to understand the physiology, pharmacology, and molecular structure of biological ligands to aid in the rational design of next-generation agents for treatment of cancer and AIDS. The Section (Weinstein) has focused on monoclonal antibodies, partly for their intrinsic biomedical interest and partly because they provide an important case study in the generic properties of biological ligands. Recent developments have included: (1) The first selective cell killing *in vivo* using an alpha emitting radioisotope conjugated to a monoclonal antibody (Black, Weinstein.); (2) Development of methods in animals and humans for selective delivery of antibodies to metastatic solid tumor and lymphoma in lymph nodes. Clinical studies (collaboration with Larson, Carrasquillo, et al.) in patients with T-cell lymphoma produced the most efficient immunological imaging of tumor cells yet achieved in humans by any non-invasive technique; (3) Quantitative modeling of the pharmacology of monoclonal antibodies by combining the global pharmacokinetics, as determined from experimental data and multi-compartmental kinetic models using the SAAM programs; the transport characteristics of tumor microvasculature; and the partial differential equations of diffusion, convection, and reaction that describe microscopic flux of ligand within tumor substance (Weinstein, Fujimori, Covell, et al.). This newly developed computer program called "PERC" is being applied to the pharmacology of a variety of endogenous and administered ligands to include explicitly such features of the problem as monovalent and bivalent binding, metabolism, and highly non-linear boundary conditions; (4) Structural analysis of the molecular correlates of antigenicity, including the development of a new algorithm ("HAL") for assessing molecular structure/function relationships in proteins and peptides (Weinstein, Viswanadhan, Guy, et al.). A "joint prediction" approach was also developed for predicting elements of protein structure (Viswanadhan).

Also the small molecule dipyridamole (Persantine) became a major subject of study in the last year when members of the Section discovered its possible use in the treatment of AIDS. Dipyridamole (DPM) is widely used as an oral agent for cardiovascular indications, and its best-defined mechanism of action is a potent inhibition of nucleoside transport into and out of cells. S. Szebeni and J.N. Weinstein found that DPM and similar inhibitors of nucleoside transport potentiate the activity of azidothymidine (AZT) and other dideoxynucleosides against human immunodeficiency virus (HIV-1) in cultured human monocytes and stimulated T-lymphocytes. Since DPM does not appear to potentiate the cytotoxic effect of AZT on human bone marrow progenitor cells *in vitro*, these findings suggested that DPM might increase the therapeutic index of AZT, and perhaps other dideoxynucleoside agents, *in vivo*. The AZT/DPM combination has now been approved for study by the AIDS Clinical Trials Group. Initial clinical studies are being planned.

Other aspects of this combination chemotherapy being studied within the Section include: (1) Mechanism: DPM blocks uptake of physiological nucleosides but not of AZT, by human monocytes (Betageri). The potentiation of AZT may thus result, in part, from decreased uptake of the nucleosides that compete with AZT for viral reverse transcriptase. Other possible mechanisms of action such as decreased CD4 expression, interferon induction, and inhibition of cyclic AMP phosphodiesterase are being examined. Parenthetically, the examination of interferon induction turned up a potentially significant finding: monocytes were identified as a source of the acid-labile interferon-alpha that has been reported as an early predictor of progression to clinical AIDS and as a contributor to the symptomatology of the disease. Preliminary polymerase chain



reaction studies appear to confirm the presence of the mRNA in HIV-infected cells; (2) Molecular structure: A structure for DPM has been computed from crystallographic coordinates. Molecular mechanics studies are being done on DPM and its analogues, in parallel with quantitative structure-activity analyses of a spectrum of similar agents (Viswanadhan); (3) Analysis of combination chemotherapy: Because no published algorithm or computer package was adequate for analysis of data on the antiviral effect of drug combinations, a new approach was developed. The new computer program, COMBO, is proving useful for the general analysis of combination chemotherapy of both cancer and AIDS.

### Membrane Structure and Function

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

### Membrane Biology

The research goals of the Membrane Biology Section (Pinto da Silva, Fujimoto, Pimenta, Ru-Long, Taylor) were pursued in four main projects. Most of the research effort was devoted to the development and application of fracture-flip and immunogold labelling/fracture-flip. These methods provide macromolecular resolution images of the ultrastructure of cell surfaces and of the distribution of cell surface antigens and receptors. The second project relates to the development and application of label-fracture, a method that provides superimposed, co-incident images of the exoplasmic fracture faces of plasma membranes and of the distribution of immunogold labelled receptors and antigens. In a third project the identification and mechanism of partition of transmembrane proteins is studied by fracture-label. The fourth project represents a continuation of our interests on membrane fusion and of

intercellular junctions. Finally, we initiated a study of the structure and cell biology of the interaction of the leprosy bacillus with the macrophage during in vitro infections. The projects of the Membrane Biology Section have benefitted from numerous collaborations.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08300-17 LTB

## PERIOD COVERED

October ., 1988 to September 30, 1989

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

SAAM, Development and Applications for Analogic Systems Realization

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

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

Other Professional Personnel:

Kevin C. Lewis, Ph.D. IRTA Staff Fellow LTB, NCI  
Peter C. Grief, M.D. Computer Programmer Analyst LTB, NCI

## COOPERATING UNITS (if any)

Dr. Ray Boston, Murdoch Univ., Australia; Dr. Charles Schwartz, Medical College of Virginia, Richmond, VA; Dr. Waldo R. Fisher, Univ. of Fla., Gainesville; Drs. H. Bryan Brewer, Daniel Rader, Carlo Gabelli, Alexander Mann, Juergen Schaefer, of

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

1.7

## PROFESSIONAL:

1.7

## 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.)

Continuing development of a computer system (SAAM) for the simulation, analysis, and modeling of bio-kinetic systems, we advanced to allow for increases in the number of compartments, components, adjustable parameters and data points. Version30 of SAAM which was developed on the VAX is available under the VMS operating system. While a generic version of SAAM30/CONSAM30 has been developed for the UNIX environment, it has not yet been optimized to run under the DEC Ultrix 3.x or the new Sun 4.x operating systems. A major effort has been applied to the development of a version of SAAM30/CONSAM30 which will execute under the DOS operating system on personal computers which make use of the Intel 80386 central processor. Because of the limitations of the DOS operating system we have made use of the Phar Lap extensions to compile and link SAAM30 and CONSAM30 on this series of computers. While SAAM30 runs quickly (compared to the VAX 8350) on these computers, it requires more than four megabytes of memory and a Weitek coprocessor. Having SAAM30 available on this class of computer makes it available to thousands of users who would ordinarily not have access to the program. While CONSAM30 (the interactive version of SAAM30) has been compiled for the 80386, the ability to support an advanced interactive graphics capabilities similar to those available on the VAX or the SUN computers remains under development. Because this effort makes use of proprietary linkers, memory extenders, and graphics drivers, licences have been obtained by the laboratory so that versions of SAAM30 and CONSAM30 can be distributed to other investigators.

Compartmental analysis was applied to plasma epinephrine (Epi) kinetics. Eight humans received a <sup>3</sup>H-Epinephrine infusion for 60 minutes. Analysis of the steady-state arterialized plasma levels of Epi and <sup>3</sup>H-Epi resulted in a two compartment model and resulted in plasma Epi appearance  $0.82 \pm 0.16$  nmol/m<sup>2</sup>/min and Epi clearance of  $1.67 \pm 0.15$  L/min/m<sup>2</sup>. The large extra-vascular mass of epinephrine was estimated which permitted new insights into the physiology and pathophysiology of adrenomedullary function in humans.

Cooperating Units (Continued):

Molecular Diseases Branch, NIH/LB; Richard E. Gregg, Squibb Inst. for Med. Res. Princeton, NJ; Dr. Ernest Schaefer, USDA Human Nutrition Center, Tufts Univ, Boston, MA; Drs. Y-Da Chen, & Gerald Reaven, Stanford Univ., Stanford, CA; Dr. Phil Taylor, Cancer Prevention Studies Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA; Dr. Blossom Patterson, Operations Research Branch, NCI; Dr. Barbara Howard, Medlantic Research Foundation, Washington D.C.; Drs. Oscar Lenaris Jeffery Halter, U of Michigan; Dr. Ba-Bie Teng & Allen Sniderman, Royal Victoria Hospital, Montreal, Quebec; Dr. Gilbert Thompson, Hammersmith Hospital, London, England; Dr. David M. Foster, University of Washington, Seattle, WA. and Dr Ahmed Kissebah, University of Wisconsin; Dr. Michael H. Green, The Pennsylvania State University. Dr's Danial Nixon, Myron Winic and Richard Costlow, Nutrition Laboratory, DCPC, NCI. Gene Barnett, Biometric Research Institute, Arlington, VA.

Project Description #1:

Project #1 The development of mathematical and computer tools for the simulation of and analysis of bio-kinetic data and the implementation of these tools within the framework of SAAM and CONSAM.

Major Findings:

(1) Computational Procedures: Continuing development of a computer system (SAAM) for the simulation, analysis, and modeling of bio-kinetic systems. In 1988-1989 yr. we advanced the SAAM/CONSAM extensions to allow for increases in the number of compartments, components and adjustable parameters having previously accomplished the increase in the number of data points which can be used with the simulator as well as an extension to 50 compartments. Version 30 of SAAM which was developed on the VAX is available under that operating system. While a generic version of SAAM30/CONSAM30 has been developed for the UNIX environment, it has not yet been optimized to run under the DEC Ultrix 3.x or the new Sun 4.x operating systems. A major effort has been applied to the development of a version of SAAM30/CONSAM30 which will execute under the DOS operating system on the series of personal computers which make use of the Intel 80386 central processor. Because of the limitations of the DOS operating system we have made use of the Phar Lap extensions to compile and link SAAM30 and CONSAM30 on this series of computers. While SAAM30 runs quickly (compared to the VAX 8350) on these computers, it requires more than four megabytes of memory and a Weitek coprocessor. Having SAAM30 available on this class of computer makes it available to thousands of users who would ordinarily not have access to the program. While CONSAM30 (the interactive version of SAAM30) has been compiled for the 80386 series of personal computers the ability to support an advanced interactive graphics capabilities similar to those available on the VAX or the SUN computers remains under development. Because this effort makes use of proprietary linkers, memory extenders, and graphics drivers, licences



have been obtained by the laboratory so that versions of SAAM30 and CONSAM30 can be distributed to other investigators.

A version of SAAM30 was developed for the UNIX-PC series of computers as more than 100 users of SAAM have this computer as their only source of computing. While this 75 compartment version of SAAM can execute on the UNIX-PC this could only be accomplished by reducing the capabilities of this version of SAAM. When this version of SAAM was combined with the interactive GKS graphics developed for the UNIX-PC, the capabilities of the computer were exceeded. Because of this a fifty compartment version of SAAM and CONSAM are under development for the UNIX-PC which will have all the numerical capabilities of SAAM30/CONSAM30. Thus the life of SAAM/CONSAM on the UNIX-PC will be extended until these users can move to SAAM30/CONSAM30 on the 80386 personal computer.

30+ C language subroutines makeup the virtual terminal graphics capability utilize the GSS-GKS C bindings on the UNIX-PC, and the Plot10-GKS Fortran bindings on the VAX under VMS. This virtual terminal graphics program has been extended by addition of the capabilities to manage segments, proportional tick sizing and color capabilities. This graphics capability has been enhanced and expanded to include support for several terminal emulators, particularly those from Plot and those found in Kermit. A version of CONSAM30 making use of a virtual terminal was developed for the VMS versions of the program. As the development of this graphics capability proceeds, the graphics program is also being migrated to Sun 3 and 4 series of workstations using the Sun GKS C bindings under SunOS 4.0. A version of GSS-GKS C bindings has been used to link the graphics routines on the 80386 series of personal computers under DOS. Because DOS is not a multitasking operating system and because CONSAM and the GSS-GKS run in different modes on the 80386 further development is required combine these two tasks. This effort may require the exploration of other GKS bindings, or multitasking extenders such as DESKview.

(2) SAAM Workshops, Distribution, & Newsletter: In the past year we have been involved in several workshops. Another two day international SAAM USERS meeting with workshops, tutorials, and presentations of work in progress was conducted in conjunction with the Resource for Kinetic Analysis. Approximately 90 participants were in attendance. This represents the fifth users meeting, previous user meetings being conducted in 1988, 1987, 1982 and 1976. SAAM29C and SAAM30/CONSAM30 were announced at this meeting. Approximately 130 investigators attended the seventh annual workshop to discuss Lipid and Lipoprotein Kinetics. Demand for this meeting is increasing because this is the principal opportunity to discuss compartmental modeling of lipoprotein modeling. This year the meeting was extended to two days because of the number of investigators who wanted to respond and present.

Hundreds of copies of the SAAM/CONSAM software have been provided to the scientific community over the past 12 months in an effort to establish other centers in the collaborative effort, as pointed out by the scientific counselors as necessary. This involves combining and confronting theorist and experimentalist with topics which can profit from the application of computer simulation and computation and further serves to obtain the best experimental data for analysis an inclusion in data bases,

such as the lipoprotein and selenium data bases. To this end we plan to continue to distribute these programs as necessary in the scientific community.

Several additional SAAMNEWS letters were written in conjunction with the Resource for Kinetic Analysis which sent each newsletter to over 600 investigators who had indicated that they had an interest in receiving such a news letter.

#### Project Description #2:

Project #2 Application of SAAM and CONSAM to the Simulation and Analysis of Bio-kinetic Data. So that the Bio-Kinetic data collected will be applicable to compartmental analysis, this effort includes chairmanship of the Radioactive Research Drug Committee where all tracer studies come under review for scientific merit. This effort also includes 800 hours of patient contact and primary responsibility for more than 30 lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI.

#### Major Findings:

(1) In collaboration with Dr. Fisher and Dr. Stacpoole detailed kinetic studies were performed in four FH subjects using an endogenous  $^3\text{H}$ -leucine tracer a precursor of nascent apolipoprotein B. Subjects were studied before and after restabilization on lovastatin, 40 mg/day in divided doses. Because these patients served as their own controls and the drug was administered in the second turnover study, the carryover effects of the drug were non-existent. Low Density Lipoprotein Cholesterol decreased  $45 \pm 9\% \pm \text{SD}$  following treatment. Following the intravenous injection of  $^3\text{H}$ -Leucine, plasma leucine, Very Low Density Lipoprotein (VLDL), and Low Density Lipoprotein (LDL) specific activities were determined over a 12 day interval. All subjects had an increase in the fractional catabolic rate and a corresponding decrease in the residence time of LDL ApoB-100. The two subjects with the longest pretreatment LDL ApoB-100 residence time and lowest production rate of apoB-100, had 2 and 3 fold increases in apoB-100 secretion in response to treatment with lovastatin; while, the two subjects with the shorter LDL residence times had little change in the apoB-100 secretion in response to therapy. In FH heterozygotes, the common response to lovastatin therapy was a decrease in a LDL-cholesterol, resulting in a lower Plasma-cholesterol. The dynamics of the control system under investigation resulted in a new steady state where the decrease in cholesterol resulted from an increased uptake of LDL particles as measured by apoB-100 residence time. In two subjects, a lowering of Plasma-cholesterol was associated with a corresponding increase in apoB-100 synthesis and secretion, and in the other two subjects, the synthesis and secretion was unchanged. This result suggests that even though this drug inhibits hepatic cholesterol synthesis, lipoprotein particle and probably cholesterol secretion into the plasma remains unchanged or is even increased.

(2) In collaboration with Dr's. Michael H. Green, Danial Nixon, Myron Winick, and Richard Costlow we are investigating Vitamin A kinetics. Despite major advances in the study of both regulatory and molecular mechanisms involved in the metabolism of vitamin A, many fundamental questions remain to be resolved regarding the role of vitamin A in the general physiological functions of growth and differentiation, reproduction, and vision. Specifically, it is important to elucidate the underlying homeostatic mechanisms that function to regulate hepatic release, plasma concentration, and recycling among the plasma, tissues, and various cell types, as well as the tissue uptake and utilization of vitamin A. An improved understanding of these mechanisms will make it possible to better estimate how certain physiological, pharmacological, or nutritionally related perturbations affect dietary requirements for the vitamin.

One approach to delineating the dynamics of metabolically significant vitamin A (retinol associated with the retinol-binding protein (RBP)/transthyretin(TTR) plasma transport complex) is to study the vitamin's whole body kinetics. For this purpose, A compartmental model was developed to describe the metabolism of this vitamin A complex in rats with low vitamin A status maintained by a low dietary intake of vitamin A (~2RE/d). Tracer and tracee data were obtained from plasma, tissue (liver, kidneys, small intestine, eyes, adrenals, testes, lungs, carcass), urine, and feces following the IV bolus injection of [<sup>3</sup>H]retinol in its physiological transport complex. The dietary protocol developed for this study resulted in animals having plasma vitamin A levels less than 10µg retinol/dl and total liver vitamin A stores of approximately 1 RE. Four compartments were used to model the plasma; 1 to describe retinol, 1 to describe the nonphysiological portion of the dose, and 2 to simulate polar metabolites derived from retinol.

The liver required 2 compartments and a delay, the carcass (small intestine, eyes, adrenals, testes, and lungs, plus remaining carcass) required 3 compartments, and the kidneys required 2. The whole-body disposal rate calculated was 1.65 RE/d with the urine and feces accounting for most of the output. The plasma turnover rate was approximately 20 RE/d which was 12 times greater than the utilization rate. This indicated that of the large amount of retinol moving through the plasma each day less than 10% of this was actually being utilized. Similarly, as compared to the whole-body utilization rate, there was a relatively high turnover rate in the kidneys (9.0 RE/d), carcass (8.2 RE/d), and liver (5.8 RE/d) coupled with a high degree of recycling of vitamin A through these tissues. Of the total vitamin A that entered the liver from all sources including the diet, approximately 86% was mobilized into the plasma. Similarly, of the vitamin A that entered the carcass, approximately 76% was returned to the plasma whereas essentially all of what entered the kidney was returned to the plasma. The present studies provide quantitative and descriptive evidence of an efficient metabolism of vitamin A from absorption through turnover and utilization in rats with very low vitamin A status. Furthermore, although their body stores of vitamin A were extremely low, these rats maintained a high rate of flow and recycling of vitamin A throughout the body.

Protocols have been written and studies begun to test the above model in the presence of other retinoids such as 4HPR and retonic acid. Results from these ongoing studies will provide quantitation of the Vit A sparing properties of these and other retinoids used in cancer chemotherapy. Further plans include using the rat as a model to test the use of stable isotopes for Vit A turnovers in preparation for a major confirmation of the model with human subjects.

(3) In collaboration with Dr. Linares; Dr. Halter, and Dr. Rosen; we used compartmental analysis to analyze plasma epinephrine (Epi) kinetics to determine whether the kinetics of distribution and metabolism of Epi could be best quantified using the isotope dilution method or a simple mathematical model. Eight human subjects received a  $^3\text{H}$ -Epinephrine infusion for 50 to 60 minutes. Analysis of the steady-state arterialized plasma levels of Epi and  $^3\text{H}$ -Epi using isotope dilution resulted in a basal plasma Epi appearance rate was  $0.87 \pm 0.11 \text{ nmol/m}^2/\text{min}$  and the basal plasma Epi clearance rate is  $1.63 \pm 0.14 \text{ L/min/M}^2$ . A biexponential plasma decay curve was achieved with a two compartment model and resulted in plasma Epi appearance  $0.82 \pm 0.16 \text{ nmol/m}^2/\text{min}$  and Epi clearance of  $1.67 \pm 0.15 \text{ L/min/m}^2$  which were not statistically different from the results of the isotope dilution. The large extra-vascular mass of epinephrine was estimated and permit new insights into the physiology and pathophysiology of adrenomedullary function in humans.

(4) In collaboration with Andre J. Jackson compartmental model for universal dosing regimens was simulated using SAAM/CONSAM. The model provides a rapid method of calculating non-uniform dosing regimens for up to a three compartmental linear model. Subject data can be curve fitted with the program to obtain individual estimates used in the facilitation of individual dosage optimization in the clinical setting. This model was used in the optimization of theophylline regimen as well as the an estimate of a new pharmacokinetic parameter,  $C_{\text{uneq}}$ .  $C_{\text{uneq}}$  is defined as the average drug concentration concentration at steady state for a drug with an irregular dosing interval.

(5) 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;  $^{14}\text{C}$  mevalonic acid, LDL free  $^3\text{H}$  or  $^{14}\text{C}$  cholesterol, VLDL free  $^3\text{H}$  and HDL free  $^3\text{H}$  or  $^{14}\text{C}$  cholesterol; frequent blood samples were then obtained for up to two days. The mass and specific activity of HDL-FC, HDL-EC,  $\beta$ -(LDL/VLDL)-FC,  $\beta$ -(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  $4.4 \mu\text{mol/min}$  respectively).



The LDL/VLDL EC data was resolved in each subject by transport of EC from HDL to LDL/VLDL (range, 7-18  $\mu\text{mol}/\text{min}$ ). 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  $\mu\text{mol}$  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.

(6) In collaboration with Drs. Ba-Bie Ting, Gilbert Thompson, Peter Kwiterovich, and Allen Sniderman LDL ApoB turnover kinetics has been re-examined. LDL ApoB kinetics have generally been analyzed by the two compartmental model of Mathues, the assumption being that all plasma LDL particles have an equal probability of being catabolized. LDL has been shown to be both structurally and kinetically heterogeneous in a wide variety of studies leading to a major contradiction of the accepted model. Accordingly a new multicompartmental model of LDL metabolism was developed and tested using data from one normal and three hyperapoB subjects. Each received a simultaneous injection of  $^{125}\text{I}$ -buoyant (L-LDL) and  $^{131}\text{I}$ -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-LDL 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-LDL. Several additional normal and familial hypercholesterolemic subjects have been examined in the past year to test the proposed model, resulting in confirmation of the new LDL model.

(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 modeling. 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,  $^{74}\text{Se}$ , was used as a label for the dose. These six sets of data have been used to realize a model for selenium metabolism.

The model developed describes the kinetics of sodium selenite metabolism in humans, based on plasma, urine, and fecal samples obtained from six subjects over a four week period following a single oral 200  $\mu\text{g}$  dose of the enriched stable isotope tracer  $^{74}\text{Se}$ . The model describes absorption distributed along the GI tract and enterohepatic recirculation. The model includes four kinetically distinct plasma components, a subsystem consisting of the liver and pancreas, and a slowly-turning-over tissue pool. For the six subjects, the ranges of mean residence times for the four plasma components are respectively: 0.2-1.1 hrs., 3-8 hrs., 9-42 hrs., and 200-285 hrs.; for the hepato-pancreatic subsystem, 4-41 days; and for the tissue pool, 115-285 days. Approximately 84% of the administered dose was absorbed and after 12 days about 65% remained in the body. The model predicts that, after 90 days, about 35% would be retained, primarily in the tissues. Separating Se metabolism into several distinct kinetic components is a first step in identifying the efficacious, nutritious, and toxic forms of the element.

In a portion of the main study sixteen subjects were each given labeled selenite. 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. Each person received a single dose in both fasting and non-fasting nutritional states. This design was chosen to allow precise measurement of any differences resulting from fasting state, while minimizing the number of subjects required. 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 on extending the pilot study model to include these data.

Over the past year we have applied this model to fasting and nonfasting main study subjects receiving inorganic selenium compounds. There appears to be a difference in the two most rapid plasma components. In a crossover study in which each subject serves as his own control, there is always the possibility of carryover. In other words, the possibility of tracer from the first study influencing the second study. Our first task was to detect and remove carryover effects, if any. A comparison of the plasma data for each subject showed that the tail of the plasma curve for the second study was higher than that for the first study. This was true regardless of fasting order.

To account for this carryover effect, we used the model to simulate the amount of tracer remaining in the body after 90 days. We estimate that about 40 % of the first dose remained at the time the second was given. Most of this was in the slowly-turning-over tissues.

The techniques of compartmental modeling give us the power to make a separation between the tracer remaining from the first study and the tracer given in the second. By retaining label in the appropriate compartments in our model, we were able to adjust for the carryover effect in estimates for the second study.

With the carryover accounted for we were free to examine differences due to feeding status. About 81% of the tracer dose was absorbed by subjects when fasting, and about 76% when non-fasting. While this difference is statistically significant at the .05 level, we do not consider it metabolically meaningful. We found no differences in cumulative levels of tracer in the urine or in the feces that corresponded to fasting status.

The plasma data told another story. The early levels of tracer in the plasma were higher when the dose was taken fasting, regardless of fasting order. To look at this difference more closely but in a preliminary way which did not take crossover effects into account, we calculated the relative differences in area under the plasma curve for fasting versus non-fasting values for each subject. That is, the fasting value less the non-fasting value, divided by the non-fasting value. These areas are derived from curves fitted to the observed data, and are not adjusted for the carryover. The differences are all positive, and decrease with time.

For all plasma components together, fasting levels are more than one and a half times as great as non-fasting levels for the first 2 hours after dosing. Over the next 6 hours this difference decreases so that, by 24 hours, there is no discernible difference between fasting and non-fasting levels.

The difference on dose day is largely due to the first plasma component. Levels are about 2 to 3 times as high for subjects when fasting as when non-fasting. This relative difference in the first plasma component persists for the entire study. However, this component only dominates the plasma curve for the first several hours.

Median levels for the second plasma component are also higher when subjects were fasting, but the differences are very small. There is no observable difference attributable to fasting status in levels of the third and fourth plasma components. These components, which are fed by the liver, pancreas, and the tissues, contain most of the plasma tracer after 24 hours.

We found that there were meaningful differences in the plasma that depended on whether the dose was given fasting or non-fasting. An explanation consistent with the model is that there is a much larger first pass effect in subjects when non-fasting than when fasting. This means that, when subjects are non-fasting, more of the dose is removed by the liver before reaching the peripheral circulation. This may be in response to increased portal flow associated with eating. We also found that there was a long-term carryover. Differences in the levels in the tail of the plasma curve reflected this effect and were not related to fasting status.

There were no metabolically meaningful differences related to fasting status in absorption, or in urinary or fecal excretion. What are some implications of these findings? Each of the four plasma pools represents a kinetically different selenium compound. These may have different mechanisms of action in relation to nutritional requirements, toxicity, and cancer prevention. Fasting state modulates the effects of the first plasma component. Suppose this component is important in cancer prevention. To maximize benefits to the

liver, the dose should be given non-fasting. To maximize benefits in the peripheral circulation, it should be given fasting.

If Se is involved in toxicity to the liver and pancreas, then the effect would be minimized if the dose were taken fasting. Using kinetic modeling techniques, we have separated the plasma into multiple components, so that each can be examined for optimal dosing conditions.

A statistically significant but nutritionally unimportant difference in absorption between the fasting,  $80.2\pm$ , and non-fasting,  $76\%$ , subjects was detected. In the feed state a larger fraction of the absorbed tracer dose is removed by the liver before appearance in the plasma. This response may be secondary to increased portal flow associated with eating. If specific plasma components prove to be important in cancer prevention,

this differentiation between the delivery of chemo-preventive selenium to the liver and peripheral tissues is modulated by the fasting status of the study subject.

A pilot model for the analysis of the kinetics organic selenium, selenomethionine has been developed using the first four studies. This model will be used to compare the kinetics of organic selenium compounds and its modulation by fasting and nonfasting status of the study subjects. These models will allow us to make population estimates of the various pharmacokinetic parameters. Finally, these estimates will be compared using analysis of variance techniques. Both main effects and interactions of form selenium and fasting state will be investigated.

Using kinetic modeling techniques, we have separated the metabolism of Se into multiple components. If, as we have suggested, the kinetically distinct plasma components represent chemically different forms of Se, each may have a specific, probably different, mechanism of action in relation to nutritional requirements, toxicity, and cancer prevention. If Se is an effective cancer prevention agent, its efficacy may not be uniform throughout the body; the amount and form reaching various organs or tissues may be of considerable research interest. Studies can be planned to identify those particular forms that show the greatest potential beneficial effect, and their particular kinetics further characterized.

It remains to take into account base line tracer levels and combine all these findings into the model to calculate a best estimate of selenite metabolism.

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Chiang CN, Barnett G. Marijuana: Pharmacokinetics and pharmacodynamics. In: Redda K, Walker C, Barnett G. (Eds) Cocaine, Marijuana, and Designer Drugs: Chemical, Pharmacological and Behavioral Aspects. CRC Press, Inc. Cleveland, Ohio 1989;113-125.

Foster DM, Boston RC, Zech LA. Kinetics of lipoprotein metabolism: Special considerations in modeling. In: Cobelli C, Mariani L. (Eds) Proceedings of the IFAC Symposium Modeling and Control in Biomedical Systems, Pergamon Press, 1988.

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Patterson BH, Levander OA, Helzlsouer K, McAdam PA, Lewis SA, Taylor PR, Veillon C, Zech LA. Human selenite metabolism: A kinetic model. Am J Physiol, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 08303-17 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Membrane Fusion Mediated by Viral Spike Glycoproteins.

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

Robert Blumenthal, Ph.D., Chief, Membrane Structure &amp; Function Section, LTB, NCI

## Other Professional Personnel:

Anu Puri, Ph.D. Visiting Associate LTB, NCI

## COOPERATING UNITS (if any)

Dr. Joel Lowy, NHBHLI; Dr. Michel Ollivon, CNRS, France; Dr. Abraham Loyter, Hebrew University, Israel; Dr. Joshua Zimmerberg, NIDDK; Dr Yi-der Chen, NIDDK; Dr F. Booy, NIDDK; Dr R.W. Doms, NIAID.

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Membrane Structure &amp; Function Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.5

PROFESSIONAL: 5.5

OTHER: 0.0

## CHECK APPROPRIATE BOX(ES)

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

## SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The research goals in the Membrane Structure and Function Section are directed towards an understanding of mechanisms of membrane fusion mediated by viral spike glycoproteins. We are specifically studying the mode of action of the G protein of Vesicular Stomatitis Virus (VSV), the HN and F proteins of Sendai Virus, and the HA protein of influenza virus. Specific topics include: i) development of fluorescent methods to study kinetics and extent of adhesion and fusion using intact and reconstituted virions, and liposomes and cells as targets; ii) development of methods to analyze reconstitution of viral spike glycoproteins; iii) functional reconstitution of viral spike glycoproteins into lipid vesicles iv) studies of mechanism based on an allosteric model: the role of ligand binding, conformational changes and cooperativity of viral spike glycoproteins in mediating membrane fusion v.) studies of the effects of modifications of viral spike glycoproteins by pH temperature, enzymes, and chemicals on their fusogenic activities vi) studies of the relationship between virus-induced membrane destabilization (permeability changes) and fusion vii.) Studies of viral entry into the cell by endocytosis using fluorescent techniques. viii.) Application of image processing using video-enhanced fluorescent microscopy controlled by a computer to analyze viral entry pathways. ix) Examination of the disposition of the fusion protein after the fusion event; x) Identification of possible fusion intermediates; xi) Development of methods to study fusion activity of mutants of viral proteins using cloned viral membrane protein sequences expressed in transfected cells; xii) Structural studies of viral proteins; xiii) Development of methods for using reconstituted viral envelopes as vehicles for specific delivery of materials into cells.

Project Description:Other Professional Personnel (continued):

Michael Clague Ph.D.	Visiting Fellow	LTB, NCI
Andreas Herrmann Ph.D.	Visiting Fellow	LTB, NCI
Christian Schoch	Visiting Fellow	LTB, NCI
Doron Kaplan Ph.D.	Visiting Scientist	LTB, NCI

Major Findings:

1. We have succeeded in monitoring the mixing of both aqueous intracellular and membrane-bound fluorescent dyes during the fusion of human red blood cells to influenza hemagglutinin (HA)-expressing fibroblasts using fluorescence spectroscopy and low-light, image-enhanced video microscopy.

2. The water-soluble fluorescent dye, N-(7-Nitrobenzofurazan-4-yl)taurine, was incorporated into intact human red blood cells. The fluorescence of the dye in the intact red blood cell was partially quenched by hemoglobin. The lipid fluorophore, octadecylrhodamine, was incorporated into the membrane of the same red blood cell at self-quenching concentrations. Fusion, which allowed movement of the water-soluble dye from the cytoplasm of the red blood cell into the HA-expressing fibroblasts, and movement of octadecylrhodamine from membranes of red blood cell to the plasma membrane of the fibroblasts, was observed by fluorescence microscopy as a spatial relocation of dyes, and monitored by spectrofluorometry as an increase in fluorescence.

3. Upon lowering the pH below 5.4, fluorescence increased after a delay of about 30s at 37 °C, reaching a maximum within 3 min. The kinetics, pH-profile, and temperature-dependence were similar for both fluorescent events measured simultaneously, indicating that influenza HA-induced fusion rapidly establishes bilayer continuity and exchange of cytoplasmic contents.

4. Movement of fluorophores between effector and target was restricted during the initial events in fusion, consistent with the opening of small junctional pore(s); HA-induced leakage of a small molecule from the target occurred concomitant with fusion.

5. In a study on the relationship between pH-induced conformational changes of viral protein and their ability to induce membrane fusion we found a marked difference between the H2 and H3 strains of influenza virus: the fusogenic activity of A/Japan/305/57 (H2 subtype) was not affected by pretreatment at low pH in contrast to X-31 (H3 subtype). Although the sequence of the fusion peptide and the location of cysteine residues are strikingly conserved, there is only about 45% sequence homology between the two strains (Gething et al, Nature, 287, 301 (1980).

6. Based on an allosteric model for viral protein-induced fusion we have defined three conformations of the influenza HA: the T (tense) state at neutral pH, and the R (relaxed) and D (desensitized) states at low pH. The T state is characterized by well-defined spike structures as observed by Cryo Electron Microscopy and by its insusceptibility to proteolytic digestion, the R state is characterized by well-defined spike structures as observed by Cryo Electron Microscopy and by its susceptibility to proteolytic digestion, and the D state is characterized by a fuzzy appearance as observed by Cryo Electron Microscopy and by its susceptibility to proteolytic digestion.

7. We find that at 37 °C the X-31 HA rapidly assumes the D state after low pH pretreatment, which is inactivated for fusion, whereas the A/Japan HA assumes the R state, which is still fusogenic. It is not clear which state is assumed during fusion.

8. Using our new spectrofluorometric assay for monitoring the the fusion of human red blood cells to influenza HA-expressing cells, we have studied the fusogenic activity of HA proteins altered in the fusion peptide sequence by site-directed mutagenesis and expressed in simian cells using SV40-HA recombinant virus vectors obtained from Dr M.J.Gething (J. Cell Biol. 102:11-23, 1986). The kinetics of fusion induced the altered HA proteins followed very well the pattern of fusogenic activity measured by cell biological techniques.

9. In order to identify the initial stages of membrane fusion induced by Vesicular Stomatitis Virus (VSV), we performed stopped flow kinetic measurements with fluorescently labeled virus attached to human erythrocyte ghosts with symmetric bilayer distributions of phospholipids. Fusion was monitored spectrofluorometrically with a 50 ms time resolution using the octadecylrhodamine dequenching assay.

10. At values close to the pH threshold for fusion a lag phase of about 2s was observed at 37 °C. This lag time decreased steeply with pH 6.05-5.8, and at pH 5.6 it was not measurable with the time resolution studied. The observed rapid fluorescence changes resulted from fusion of virus bound to the target, and the time lags were not due to association-dissociation reactions between virus and target. The initial rates of fusion showed similar steep dependence on pH. For a given pH value temperature dependence of delays and rates was similar.

11. Using SAAM, the results were fitted to a multistate model similar to those resulting from ion channel gating kinetics. The model allows certain conclusions to be drawn about the role of cooperativity and conformational changes in viral spike glycoprotein-mediated membrane fusion.



Publications:

Chejanovsky N, Nussbaum O, Loyter A, Blumenthal R. Fusion of enveloped viruses with biological membranes: Fluorescence dequenching studies. *Subcell Biochem* 1988;13:415-457.

Blumenthal R. Membrane fusion mediated by viral spike glycoproteins: A model based on cooperativity and conformational changes. *Studia Biophysica* 1988;127:147-154.

Grimaldi S, Verna R, Puri A, Morris SJ, Blumenthal R. Fusion of vesicular stomatitis virus with human red blood cell membranes: The role of phospholipid distribution. In: Jorgensen PL, Verna R. (Eds) *Advances in Biotechnology of Membrane ion Transport*. Serono Symposia Publications, 1988;51:197-211.

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Sarkar DP, Blumenthal R. The role of the target membrane structure in fusion with sendai virus. *Membrane Biochemistry*, in press.

Harris AL, Walter A, Zimmerberg J. Transport-specific isolation of large channels reconstituted into lipid vesicles. *J Membrane Biol*, in press.

Walter A, Eidelman O, Ollivon M, Blumenthal R. Functional reconstitution of viral envelopes. In: Wilschut J, Hoekstra D. (Eds) *Cellular Membrane Fusion: Fundamental Mechanisms and Applications of Membrane Fusion Techniques*. Marcel Dekker, Inc., in press.

Miguel MG, Eidelman O, Ollivon M, Walter A. Temperature dependence of the vesicle-micelle transition of egg phosphatidylcholine and octyl glucoside biochemistry, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 08320-14 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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 L. Jernigan, Ph.D., Theoretical Physical Chemist LTB, NCI

## Other Professional Personnel:

Shou-ping Jiang Visiting Scientist LTB, NCI

H. Robert Guy, Ph.D. Sr. Staff Fellow LTB, NCI

Kai-Li Ting, Ph.D. Computer Programmer LTB, NCI

## COOPERATING UNITS (if any)

Dr. J. Ferretti, Laboratory of Chemistry, NIH/BL

Dr. Arieh Ben-Naim, Dept. Physical Chemistry, Hebrew Univ., Jerusalem, Israel

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

1.5

## PROFESSIONAL:

1.3

## 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.)

The solution conformations of two tachykinins, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH<sub>2</sub>), physalaemin (pGln-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH<sub>2</sub>), senktidä (Asp-Phe-Phe-Gly-Leu-Met-NH<sub>2</sub>) and septide (Asp-Phe-Phe-Pro-Leu-Met-NH<sub>2</sub>) have been studied here with a combination of NMR 2-dimensional NOE'S and semi-empirical energy calculations. The number of possible conformations is extremely large, and requires a large computational time to locate the low energy conformations of these peptides. Energy calculations are carried out for fragments to find low energy conformations by energy refinement. Then, these are used as possible starting conformations for the whole molecule. Thus, the starting conformations of the whole molecule are chosen by combining the low energy conformations of each fragment. Following this, the total conformational energies are calculated and energy refinements carried out. Conformations are then selected that are consistent with the NMR 2-dimensional NOE data.

Project Description:Major Findings:

In water, substance P and physalaemin prefer to be in an extended conformation. However, in methanol, their conformations are a mixture of  $\beta$ -turn conformations in dynamic equilibrium. Solvent titration data and chemical shift temperature coefficients complement the NMR estimates of interproton distances by assisting in locating hydrogen bonds and identifying dominant conformational states. The C-terminal tetrapeptide appears to have the same conformation for both substance P and physalaemin. In physalaemin, the middle of the peptide is constrained by the formation of a salt bridge.

In methanol, the lowest energy conformation of senktide is found to be an  $\alpha$  helical structure with additional hydrogen bonds between the carbonyl oxygen of Phe2 and the amide groups of Gly and Leu, and other possible hydrogen bonds, such as between CO(Phe3)-NH(NH<sub>2</sub>) and CO(Asp)-NH(Leu). The NH group of Phe3 points toward the outside in its low energy conformation. The planes of the aromatic rings of Phe2 and Phe3 point in different directions and are almost perpendicular to each other. All tachykinins have the Gly-Leu-Met-NH<sub>2</sub> fragment in common and Substance P (SP) has Phe-Phe-Gly-Leu-Met-NH<sub>2</sub> fragment in the C terminal sequence. The  $\alpha$  helical conformation is one of the lowest energy conformations for senktide. Substance P may be in  $\alpha$  helical conformation at the Phe-Phe-Gly-Leu-Met-NH<sub>2</sub> on its C terminal end, and at least partially  $\alpha$  helical conformations are common to all tachykinins.

The lowest energy conformation for septide is very different from senktide because of the replacement of glycine in senktide with proline in septide. None of the low energy conformations have an  $\alpha$  conformation just before the proline. But  $\beta$  conformation and some others followed by proline exist among low energy conformations.

PUBLICATIONS:

Ben-Naim A, Ting K-L, Jernigan RL. Solvation thermodynamics of biopolymers I. Separation of the volume and surface interactions with estimates of the volume and surface interactions. Biopolymers, in press.

Ben-Naim A, Ting K-L, Jernigan RL. Solvation thermodynamics of biopolymers II. Correlations between functional groups. Biopolymers, in press.

Ben-Naim A, Ting K-L, Jernigan RL. Solvent effect on binding thermodynamics of biopolymers. Biopolymers, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 08335-13 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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. Chief, Theoretical Immunology Section, LTB, NCI

## Other Professional Personnel:

Janos Szebeni, M.D. Visiting Associate LTB, NCI  
 Gurupadappa Betageri, Ph.D. Visiting Fellow LTB, NCI

## COOPERATING UNITS (if any)

Drs. Sharon M. and Larry M. Wahl, NIDR; Drs. Mika Popovic and Suzanne Gartner, LTCB, DCE; R. J. Parker, Office of Director, DCE

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Theoretical Immunology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

0

## CHECK APPROPRIATE BOXES)

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

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

We have studied three conceptually different ways of "targeting" liposomes:

(1) Antibody-mediated targeting. We found that antibody-bearing liposomes bind in large numbers to cells which bear the appropriate antigen. However, the bound liposomes are internalized only if endocytosis is possible. Upon endocytosis, liposome-entrapped methotrexate (MTX) can escape from the endocytic apparatus and bind to cytoplasmic dihydrofolate reductase, inhibiting growth of the cell. In the course of these studies, we developed the first heterobifunctional method for coupling antibody to liposomes. Current studies are directed toward HIV-infected cells.

(2) Physical targeting. We 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 or cis platinum to mouse tumors in vivo and inhibit their growth.

(3) Compartmental targeting. We have demonstrated the delivery of liposomes and entrapped drug to lymph nodes after subcutaneous and intraperitoneal injection and have determined cellular sites of localization. These studies have been extended to antibody-bearing liposomes.

These strategies are being applied to problems in the therapy of cancer and AIDS. With respect to AIDS, we have formulated liposomes containing anti-viral drugs (dideoxynucleoside triphosphates) which do not ordinarily enter cells but which can be carried into monocyte/macrophages by the liposomes. Alternatively antibody is attached to the liposomes by our previously developed methods for delivery to targeted cell types.



Project DescriptionMajor Findings:

Our objectives are 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) target HIV-infected cells.

Objective 1: (a) Bivalent antibody selectively binds TNP-bearing liposomes to TNP-bearing lymphocytes, but the binding does not increase delivery of liposome contents to the cell interior (beyond the amount internalized spontaneously). (b) Endogenous surface IgA on cells of the murine myeloma MOPC 315 can bind liposomes bearing the appropriate hapten (DNP) to the cell surface. However, as in the lymphocyte system, binding does not increase delivery to the cytoplasm. (c) Lipid vesicles containing fluorescent molecules are potentially useful as markers for sparse or low-affinity cell surface determinants. They can be made to contain many fluorophore molecules, thus amplifying the signal. They give very low-nonspecific background since the fluorophore is sequestered, and appropriate lipids are not very "sticky". (d) Liposomes are removed from the circulation of a mouse much faster if the mouse carries a myeloma secreting antibody to a hapten on the liposomes. (e) Antibody-mediated binding of methotrexate-containing vesicles to MOPC 315 and TEPC 15 myeloma cells did not lead to entry of drug into the cells and inhibition of their metabolism. (f) IGG opsonized DNP-vesicles are bound in large numbers to Fc receptor-bearing cells (murine P388D<sub>1</sub>), and are then endocytosed. Liposome-encapsulated MTX can escape the phagolysosomal 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) propionate. This method of coupling results in only minimal aggregation and little leakage of vesicle contents. Liposomes bearing covalently coupled mouse monoclonal antibody against human  $\alpha_2$ -microglobulin bind specifically to human cells, but not to mouse cells. (h) Liposomes bearing antibody directed against the class I histocompatibility antigen H-2K<sup>k</sup> selectively kill class I-bearing cells. Work with such liposomes and with immunotoxins has led us to a new cell biological hypothesis, the "neutral bypass" (see also project no. Z01 CB 08367-03 LT8).

Objective 2: (a) Small unilamellar 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<sub>c</sub> but release their contents within a few seconds upon passage through T<sub>c</sub> 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 continuously 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.

Objective 5: (a) Liposomes containing dideoxycytidine triphosphate were formulated and characterized for leakage rate, size distribution, and composition. (b) The liposomes were found to inhibit HIV-1 replication in cultured monocyte/macrophages as potently as did free drug. (c) Pilot studies of i.v. injection into mice with BMS viral infection were conducted with H. Morse, NIAID. (d) Murine IgG<sub>2a</sub> was corralently bound to the liposomes and found to increase their uptake by monocyte/macrophages.

#### Publications:

Quill H, Carlson L, Fox BS, Weinstein JN, and Schwartz, RH. Optimization of antigen presentation to T cell hybridomas by purified Ia molecules in planar membranes: Ia molecule polymorphism determines the antigenic fine specificity of the response to cytochrome c peptides. *J. Immunol. Methods*, 1987;98:29-41.

Barbet J, Black CDV, Holton OD III, Weinstein JN. Specific toxicity to activated T and B lymphocytes of a ricin A immunotoxin directed against the

class I MHC antigen H-2 K. J. Antibody, Immunoconjugates and  
Radiopharmaceuticals 1988;1:169-180.

Mitchell MS, Weinstein JN. Biotechnology products in cancer therapy. Cancer  
Research 1988;47:223-225.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08341-11 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Structure/Function Relationships in Molecules Important to Treatment of Cancer &amp; AIDS

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

John N. Weinstein, M.D., Ph.D.

Chief, Theoretical Immunology  
Section

LTB, NCI

## Other Professional Personnel:

H. Robert Guy, Ph.D.

Senior Staff Fellow

LTB, NCI

Kai-Li Ting, Ph.D.

Computer Programmer

LTB, NCI

Vellarkad Viswanadhan

Biotechnology Fellow

LTB, NCI

## COOPERATING UNITS (if any)

Drs. J. Segrest &amp; Anantharamaiah, U. Alabama, Birmingham; Pat Elwood, MB, NCI

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Theoretical Immunology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

1.0

## PROFESSIONAL:

1.0

## OTHER:

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.)

Statistical mechanical algorithms (HAL, HALP, HALCO) were devised for evaluating amphipathic helical structures and more general structure-function relationships in proteins and peptides. These computational approaches are being used to define issues of structure and possible immunogenicity with respect to HLA antigens and to the envelope polyprotein of HIV.

Also developed was a new joint prediction approach to protein secondary structure. The method combines neural network, information theory, sequence homology, hydrophobicity, loop potential, and amphipathicity criteria (including HAL). We have applied it to structure predictions on the human and bovine folate binding proteins, which are important in the cellular handling of folates and antitumor drugs such as methotrexate.

Quantitative structure/activity relationship (QSAR) studies are being done on a set of 20 inhibitors of nucleoside transport into cells. In parallel, a molecular mechanics study is being done on analogues of the unusual nucleoside transport inhibitor dipyrindamole. Our group has found that dipyrindamole (and at least one other nucleoside transport inhibitor) potentiates the activity of azidothymidine against HIV-1 replication in cultured monocyte/macrophages and T-lymphocytes (see report Z01 CB 08392-01 LTB). Animal studies are in progress and clinical trials are being planned, in parallel with continuing attempts to understand the mechanisms of action and molecular structure/function relationships.



Project Description:Major Findings:

(1) Development of the HAL (Helical Amphipathicity Locator) algorithm and computer program set for analysis of structure/function relationships in proteins. Related packages further developed during the last year include HALP, HALPSTAT, HALCOMP, COPHAL and HALCO. (12) Application of these programs to the problem of finding T-cell antigenic peptides and other structural features of the HIV envelope protein. (13) Predicted peptides have been synthesized and have undergone physical chemical and immunological testing.

(2) Use of HAL to determine binding sites on proteins that interact with suramine. Analysis of the structure of suramine.

(3) Development of a new joint prediction approach to protein secondary structure. The method combines neural network, information theory, sequence homology, hydrophobicity, loop potential, and amphipathicity criteria (including HAL). We have applied it to structure predictions on the human and bovine folate binding proteins, which are important in the cellular handling of folates and antitumor drugs such as methotrexate.

(4) Quantitative structure - activity study of certain synthetic nucleotide inhibitors of nucleotide transport in animal cells.

(5) Conformational analysis of DPM and related analogs using the MM2(87) molecular mechanics package.

Publications:

Quill H, Carlson L, Fox BS, Weinstein JN, Schwartz, RH. Optimization of antigen presentation to T cell hybridomas by purified Ia molecules in planar membranes: Ia molecule polymorphism determines the antigenic fine specificity of the response to cytochrome-c peptides. J Immunol Methods 1987;98:29-41.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

E01 CB 08359-08 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Monoclonal Antibodies in the Lymphatics for Diagnosis and Therapy of Tumors

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

John N. Weinstein, M.D., Ph.D. Chief, Theoretical Immunology Section LTB, NCI

## COOPERATING UNITS (if any)

Dr. A. Keenan, Dr. S.M. Larson, LHM, CC; Dr. R. Parker, Dr. S. Sieber, DCCP; Dr. L. Liotta, Dr. G. Bryant, LP, DCBD; Dr. M. Lotze, Dr. R. Rosenberg, SB, DCT; Dr. J. Mulshine, NCI-NMOB, DCT.

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Theoretical Immunology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

## PROFESSIONAL

## OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

We have defined a new approach to the use of monoclonal antibodies for diagnosis and therapy of tumor in lymph nodes: delivery to the nodes via lymphatic vessels after subcutaneous injection. To establish a firm pharmacokinetic basis for this approach, we first studied antibodies to normal cell types in the mouse lymph node. In vitro binding characteristics were combined with in vivo pharmacological parameters to develop a quantitative understanding of the delivery process using the SAAM computer modeling system. Armed with that background information, we then demonstrated and analyzed specific uptake in lymph node metastases of a guinea pig tumor. The approach was extended to include endoscopic techniques for reaching lymph node groups not accessible by subcutaneous injection. Imaging studies were followed up with attempts at therapy. For diagnosis of early metastatic tumor in the nodes, the lymphatic route can provide higher sensitivity, lower background, lower systemic toxicity, and faster localization than the intravenous route. It will also minimize the problem of cross-reactivity with antigen present on normal tissues. This technique has been applied to detection of lymph node metastases in clinical stage II malignant melanoma and cutaneous T-cell lymphoma (CTCL) (with very good results). Similar protocols have been approved for breast carcinoma, small cell lung carcinoma, and non-small cell lung carcinoma. Our studies of CTCL produced the most efficient antigen-specific imaging yet achieved in humans by any technique. The 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. This work on "immunolymphoscintigraphy" has been followed up clinically by others, who recently published encouraging results on diagnosis of axillary metastases of breast cancer.

This project was inactive in 1988-9 except for writing and publications.

Project Description:Major Findings:

(i) Establish a quantitative basis (experimental and theoretical) for lymphatic delivery of monoclonal antibody to lymph nodes; (ii) Use that information for optimization of diagnostic imaging of animal tumors; (iii) Treat the tumors with radio-labeled and/or toxin-labeled antibodies (using experimental parameters obtained from the diagnostic studies); (iv) Extend the principles of lymphatic immunodiagnosis and immunotherapy to the clinical setting; (v) Extend our theoretical and experimental models to the intravenous administration of monoclonals; (vi) Apply the lymphatic approach to detection of lymph node metastases of malignant melanoma, breast carcinoma, lymphoma, non-small cell lung carcinoma, and other malignancies; (vii) Extend the lymphatic delivery of monoclonal antibodies to therapeutic modalities; (viii) Investigate the lymphatic pharmacology of immunization and toleragenesis.

## Antibodies directed against normal cells:

(1) Antibodies against normal cell types (in the mouse and guinea pig) can be delivered with high efficiency from subcutaneous injection sites to regional lymph nodes. The normal nodes can be imaged with the gamma camera if the antibodies are radiolabeled with I-125 or I-131. (2) Injections can be made so as to label all cells of the target population in the node. (3) Pharmacokinetic experiments and modeling indicate that correct choice of dose, volume, osmolarity, and site of injection are all important if efficiency of the imaging technique is to be maximized. These findings suggest clinical use of antibodies against normal lymphoid cells as an alternative to colloid lymphoscintigraphy for diagnostic imaging of lymph nodes.

## Antibodies directed against tumor cells:

(1) Antitumor antibodies can be delivered with high efficiency from subcutaneous injection sites to regional lymph nodes (in the guinea pig). (2) Early metastatic tumor in the lymph nodes is selectively labeled with the antibody, and gamma camera images of the tumor deposits can be obtained. (3) The smallest amounts of tumor imaged in this way are about 3 mg (much smaller than has been detected by i.v. monoclonal antibodies or other non-invasive methods of detection). (3) The double-label experimental design developed for animal studies is appropriate for use in clinical trials of lymphatic delivery of monoclonal antibodies. (4) Antibodies against normal lymphoid cells can be used in tandem with antibodies against tumor. The former indicates gross characteristics of the node and/or its immunological status; the latter detects metastatic tumor with high sensitivity. (5) Subcutaneous injections of T101 antibody in patients with cutaneous T-cell lymphoma (in collaboration with the Clinical Center Nuclear Medicine Department, NIH/Naval Medical Oncology Branch, and others) produced the most efficient, antigen-specific imaging yet achieved in human subjects. (6) Extension of the lymphatic technique to reach node groups inaccessible from subcutaneous injection sites.

In particular, antibodies injected via a bronchoscope into peri-bronchial tissue labeled the pulmonary lymph nodes efficiently and selectively in experimental animals. The technique is being incorporated into protocols for human lung cancer. (7) Development of kinetic models for the pharmacology of monoclonal antibodies in animals (see Report No. 4) and humans; (8) Selective depletion of B-lymphocyte from regional lymph nodes of mice injected with conjugates of an alpha emitter (Bi-212) and an IgG reactive with murine B-cells. This was the first demonstration of selective cell killing in vivo with an alpha emitter/antibody conjugate.

Publications:

Weinstein JN, Eger RR, Covell DG, Black CDV, Mulshine J, Carrasquillo JA, Larson SM, Keenan AM. The pharmacology of monoclonal antibodies. *Annals NY Acad Sci*, 1987;507:199-210.

Black CDV, Atcher RW, Barbet J, Brechbiel MW, Holton OD III, Hines JJ, Gansow OA, Weinstein JN. Selective ablation of B lymphocytes in vivo by an alpha emitter, bismuth-212, chelated to a monoclonal antibody. *J. Antibody, Immunoconjugates and Radiopharmaceuticals*, 1988;1:43-53.

Black CDV, Kroczek RA, Barbet J, Weinstein JN, Shevach EM. Induction of IL-2 receptor expression in vivo: Response to concanavalin A. *Cellular Immunol.*, 1988;111:420-432.

Weinstein JN. Immunolymphoscintigraphy and other regional applications of monoclonal antibodies In: Zalutsky M ed. *Antibodies in Radiodiagnosis and Therapy*, Boca Raton, FL: CRC Press, 1988:153-167.

Kroczek RA, Black CDV, Barbet J, Shevach EM. Induction of IL-2 expression in vivo. Response to allogeneic cells. *Transplantation*, 1988, in press.

Kroczek RA, Black CDV, Barbet J, Shevach EM. Mechanism of action of Cyclosporin A in vivo 1 Cyclosporin A fails to inhibit T lymphocyte activation in response to alloantigens, *J of Immunology*, 1988, in press.

Weinstein JN. Antibody lymphoscintigraphy. In: Goldenberg DM, ed. *Cancer imaging with radiolabeled antibodies*, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 08363-07 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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.

Senior Staff Fellow

LTB, NCI

Other Professional Personnel:

G. Raghunathan

Visiting Fellow

LTB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

2.0

## PROFESSIONAL

2.0

## OTHER:

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.)

The primary goal of this project is to develop methods to predict structures of membrane proteins from their sequences and available experimental data and to use these methods to develop structural models of specific membrane proteins. The simplest structures we have been analyzing are a series of peptides (melittin,  $\delta$  lysin, magainin, PGLa, and pardaxin) that form amphipathic  $\alpha$  helices that aggregate in different ways dependent upon the environment surrounding the peptide. These studies involve combining computer graphics techniques with energy calculations by the program CHARMM. To test these methods we developed a model of the crystal structure of  $\delta$  lysin. This peptide has been crystalized and the unit cell dimensions have been determined; however, its precise structure is not yet known. Magainin and PGLa are potent antimicrobial peptides found in Xenopus skin. The antimicrobial activity of these peptides and their ability to permeablize lipid vesicles and lyse cells is much greater when both peptides are present. We developed structural models of how magainin and PGLa can self aggregate and aggregate with each other in order to understand the molecular basis for this synergistic effect.

We have also continued to develop structural models of larger integral membrane proteins. The protein families that we are currently analyzing are transmitter-activated channels (nicotinic acetylcholine, GABA<sub>A</sub>, and glycine receptors), voltage activated channels (sodium, calcium, and potassium channels), receptors that activate G proteins (adrenergic, muscarinic acetylcholine, serotonin, dopamine, substance K, and opsin receptors), and gap junction channels.

Project Description:Major Findings:

It is important to test new methods of predicting protein structure in a manner that cannot be biased by knowing the structure. We developed a model of  $\delta$  lysin and how it aggregates in the crystal as a test of our methods for combining computer graphic procedures with energy minimization methods. This peptide has been crystallized but the crystal structure has not yet been determined.

The type of synergistic effects on membranes or microbes caused by magainin and PGLa has not been previously observed for peptides. The models we have developed for how these peptides aggregate with themselves and with each other in membranes explains why some sequential changes in magainin potentiate the magainin antimicrobial effect but prevent the synergistic effect whereas other changes inhibit the antimicrobial effect but potentiate the antimicrobial effect. We are collaborating with Michael Zasloff's group to test additional aspects of the model.

Proteins that act by activating G-proteins are perhaps the largest superfamily of membrane proteins involved in transduction of signals. Craig Ventor's group has determined several sequences for proteins in this superfamily are performing mutagenesis experiments on these proteins. We are collaborating with them to develop structural models of several proteins within this superfamily. We have tentative models of the positions and orientation of transmembrane  $\alpha$  helices and are working to make the models more precise.

We have continued our work on transmitter-activated and voltage-activated channels. Our original model of the sodium channel has been modified slightly on the basis of new sequences and has been extended to include potassium and calcium channels. We have developed a new model for the transmitter activated channels.

Sequences have been determined recently for several gap junction channel proteins. We have developed a tentative model of the transmembrane region of these channels that is consistent with the X-ray diffraction and electron microscopy studies that have been performed on these proteins.

Much of our recent work on large integral membrane proteins is based in part on information obtained by comparing homologous sequences. We are developing more quantitative methods of using this information in model building and have demonstrated that the concepts we used in modeling membrane proteins are valid for soluble globin proteins for which the structure is known.

Publications:

Guy HR. Models of voltage- and transmitter-activated channels based on their amino acid sequences. In: Pasternak CA. (Ed) Monovalent Cations in Biological Systems. CRC Press, in press.

Guy HR, Raghunathan G. Structural models for membrane insertion and channel formation by antiparallel  $\alpha$  helical membrane peptides. In: Pullman A, Jortner J, Pullman, B. (Eds) Transport Through Membranes: Carriers, Channels, and Pumps. Jerusalem Symposium on Quantum and Biochemistry, 1989;21:369-379.

Guy HR, Raghunathan G. Structural models of alpha helical membrane peptides and the GABA receptor channel. In: Computer-Assisted Modeling of Receptor-ligand Interactions: Theoretical Aspects and Applications to Drug Design. Alan R. Liss, Inc., 1989;231-243.

Guy HR. Molecular models for voltage activated channels. Proceedings of International Conference on: Molecular and Cellular Mechanisms of Antiarrhythmic Agents. In press.

Guy HR. A model relating the sodium channel's structure to its function. In: Agnew W, Sigworth F, Claudio T. (Eds) Molecular Biology of Ion Channels. Current Topics in Membrane Transport, 1988;33:289-308

Williams WV, Guy HR, Rubin DH, Robey F, Myers JN, Kieber-Emmons T, Weiner DB, Green MI. Sequence of the cell attachment site of reovirus type 3 and modeling of its three-dimensional structure. Proc Natl Acad Sci USA 1988;85:6488-6492.

Williams WV, Guy HR, Weiner D, Rubin D, Green MI. Structure of the neutralization epitope of the reovirus type-3 hemagglutinin. Vaccines 1988;88:25-28.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08365-07 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Structural Properties of T-cell Antigenic Sites

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

Robert L. Jernigan, Ph.D. Theoretical Physical Chemist LTB, NCI

## Other Professional Personnel

Hanan Margalit, Ph.D.	Visiting Associate	LTB, NCI
John L. Spouge, M.D., Ph.D.	Visiting Associate	LTB, NCI
David G. Covell, Ph.D.	Expert	LTB, NCI

## COOPERATING UNITS (if any)

Jay A. Berzofsky, Metabolism Branch, DC3D, NCI; James L. Cornette, Department of Mathematics, Iowa State University; Charles DeLisi, Mount Sinai 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

1.0

## PROFESSIONAL

1.0

## OTHER:

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.)

Intrinsic sequence features are important for T-cell recognition. T lymphocytes (T cells) recognize only a small number of segments (antigenic sites) of a foreign protein, and only when the segment is presented on the surface of an antigen presenting cell in association with a major histocompatibility (MHC) molecule. Previously, by statistical methods we found properties of the known antigenic sites that distinguish them from the rest of the protein. The property that was strongly correlated with T-cell antigenicity was  $\alpha$ -helical amphipaticity, and we developed a predictive algorithm, based mainly on this property. We are currently interested in studying the sequence features that determine the MHC restriction. We hope to incorporate those properties into the predictive algorithm, so that in addition to prediction of immunodominance it will identify the MHC restriction of a peptide. The ability to better predict T-cell sites from the primary sequence will be important for the design of synthetic and recombinant vaccines. [See also report Z01 CB 04020-09 MET of Jay A. Berzofsky, with whom this work is closely coordinated.]

Our approach has been to simulate the complex of antigen-MHC class II molecule, based on the known structure and published coordinates of the class I molecule. By repeating the analysis for all antigens that are restricted to a specific MHC, we can look for consistent rules, and then test our hypothesis by simulating the same antigens with other MHC types for which they are known not to be restricted. Recently, we have also applied a new method, developed for protein folding problems, to generate all possible conformations of an amino acid sequence in the limited space of the binding region.



Project Description:Major Findings:

As derived earlier from studies of immunodominant sites in proteins, the following properties correlate with a peptide being a T cell immunodominant site: the ability to fold into an  $\alpha$ -helix, the resulting helix being amphipathic, having COOH-terminal lysines, and not being segmentally amphipathic. Variant peptide studies allow another type of statistical evaluation, and preliminary results confirm the role of  $\alpha$ -helical amphipathicity as a correlate of peptide antigenicity. In addition to the properties that make a site immunodominant, we are studying the sequence features that determine the MHC restriction. For this analysis we use two approaches. Both of them use the known three dimensional structure of class I molecule to model the structure of class II molecule, using sequence alignment methods. The structure contains two  $\alpha$  helices with a  $\beta$  sheet floor, forming a cleft within which the peptide is believed to interact with the MHC molecule. We are looking for the best placement of the peptide in this cleft, trying to identify peptide and MHC residues that are crucial for the interaction. By the first approach we model the peptide as an  $\alpha$  helix. There are many possible conformations for complexes formed between the two  $\alpha$  helices and an antigenic  $\alpha$  helix. The likelihood of each conformation is evaluated so that the most favorable ones can be determined. By the second approach no specific structure is assumed for the peptide but its structure is a result of the simulation. In the second approach a set of points on a cubic lattice is used both to restrict the position of the peptide to the binding region, as well as for the generation of all of its conformations consistent with this constraint. One residue of the peptide is tethered to the protein, in this case, based upon hydrophobicity patches of the protein. The large MHC protein is taken to be rigid. Typically, a few million conformations of the peptide are generated. Subsequently, the energies of all these conformations are evaluated in terms of residue-specific, pairwise contact energies that favor non-bonded, hydrophobic interactions. The results give some indication of the relative influence of the intra-molecular peptide energy and the inter-molecular binding energy. The simulations are done for each Ia type and the data base of its known antigens separately. From these models, specific sequence features that determine the MHC restriction can be studied and it is hoped that the properties determining immunodominance of antigenic sites can be understood at a basic level. Understanding the biochemistry of these structures will be valuable for the design of synthetic or recombinant vaccines.

Several schemes are now available for predicting immunodominant T cell sites: these schemes have commercial importance as predictors of possible peptide vaccines. A statistical evaluation of these schemes has three objectives: to encourage the further collection of unbiased data, to determine the best scheme, and to have a criterion by which to judge future schemes. Evaluation of alternate schemes is in progress.

Publications:

Cornette JL, Margalit H, DeLisi C, Berzofsky JA. Concepts and methods in the identification of T cell epitopes and their use in the construction of synthetic vaccines. *Methods in Enzymology*, in press.

Margalit H, DeLisi C, Berzofsky JA. Computer predictions of T cell epitopes. In: Woodrow GC, Levine MM, Eds. *The Molecular Approach to New and Improved Vaccines*. New York: Marcel Dekker, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08366-06 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

The Percolation of Monoclonal Antibodies into Tumors

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

John N. Weinstein, M.D., Ph.D., Chief, Theoretical Immunology Section, LTB, NCI

Other Professional Personnel:

Kenji Fujimori, M.D.

Visiting Fellow

LTB, NCI

## COOPERATING UNITS (if any)

Dr. L. Liotta, LP, DCBD; Dr. John Fletcher, LAS, DCRT

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Theoretical Immunology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS

0.9

## PROFESSIONAL

0.9

## OTHER:

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 that splice together the global pharmacology and the microscopic percolation process. Significant predictions thus far include the following: (1) The diffusion coefficient and/or hydraulic conductivity may limit flux of antitumor Ig through tumors. (2) The flux of non-binding control Ig is much less likely to be limited by diffusion or convection. Nonspecific Ig's penetrate more deeply and more quickly into the tumor. (3) Even with saturable binding (but not metabolism), the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. (4) Metabolism will decrease the relative "C times T" exposure of cells farther from the source. This may be a major barrier to effective treatment of solid tumors with ligand molecules. (5) Most interesting, antibodies with low affinity may be preferable at a given dose to those with high affinity for some therapeutic applications.

We plan to test predictions of the model using micrometastases of human melanoma in nude mice. The distribution of antibody will be determined by fluorescence techniques and autoradiography. Concepts arising from this study are being applied to the design of clinical studies with monoclonal antibodies.

In addition to the investigations of immunoglobulin and other ligands (e.g., soluble CD4) as administered agents, we are considering the physiology of endogenous molecular species including the lymphokines and growth factors.

Project DescriptionMajor Findings:

(1) For physiologically reasonable ranges of parameters, the diffusion coefficient and/or hydraulic conductivity may limit flux of antitumor antibodies through tumors; (2) The flux of non-binding control antibody is much less likely to be limited by the rate of diffusion or convection through the tumor. Such antibodies are predicted to penetrate more deeply and more quickly into the tumor; (3) In the presence of saturable binding but not metabolism, the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. However, the period of exposure will be later for cells farther from the source of antibody; (4) Metabolism will decrease the relative CxT exposure of cells farther from the source. This may be a major barrier to effective treatment of solid tumors with ligand molecules; (5) Antibodies of low affinity may be preferable to those of high affinity at a given dose for some purposes, e.g. in therapy with alpha-emitting conjugates or toxin conjugates. (6) Quantitative, but not qualitative, changes in predicted behavior are seen as the geometry changes from Cartesian to cylindrical (for cords of tumor cells surrounding a central blood vessel) to spherical (for flux into a nodule of cells); (7) the characteristics for IgG, F(ab')<sub>2</sub>, and Fab can be compared; (8) Bivalent binding can be simulated; (9) the global pharmacology can be integrated with the percolation calculations; (10) the computer program package developed ("PERC") is broadly capable of handling various ligands of differing valence.

Publications:

Weinstein, JN. Immunolymphoscintigraphy and other regional applications of monoclonal antibodies. In: Zalutsky M ed. Antibodies in Radiodiagnosis and Therapy, Boca Raton, FL, CRC Press, 1988;153-167.

Weinstein JN, Eger RR, Covell DG, Black CDV, Mulshine J, Carrasquillo JA Larson SM, Keenan AM. The pharmacology of monoclonal antibodies. Annals NY Acad Sci 1987;507:199-210.

Black CDV, Atcher RW, Barbet J, Brechbiel MW, Holton OD III, Hines JJ Gansow OA, Weinstein JN. Selective ablation of B lymphocytes in vivo by an alpha emitter, <sup>212</sup>bismuth, chelated to a monoclonal antibody. J Antibody, Immunoconjugates and Radiopharmaceuticals 1988;1:43-53.

Fujimori K, Weinstein JN. Pharmacokinetics and organ targeting of monoclonal antibodies. Advances in Immunopharmacology 1989;4:127-129.

Fujimori K, Covell DG, Fletcher JE, Weinstein JN. A modeling analysis of IgG, F(ab')<sub>2</sub>, and Fab in tumors. Cancer Research, in press.

Weinstein JN. Antibody lymphoscintigraphy. In: Goldenberg DM, ed. Cancer imaging with radiolabeled antibodies, in press.

Weinstein JN, Fujimori K. Antibody-mediated drug delivery. In: Poste, ed. Protein Design and the Development of New Therapeutics and Vaccines, in press.

Weinstein JN, Fujimori K. Predictive pharmacology of monoclonal antibodies. Cancer Therapy: New Trends. in press. 659



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 C3 08367-06 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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)

John N. Weinstein, M.D., Ph.D., Chief, Theoretical Immunology Section, LTB, NCI

## COOPERATING UNITS (if any)

Dr. Christopher D.V. Black, ROB, DCT, NCI; 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.

0.1

## PROFESSIONAL

0.1

## OTHER:

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.)

Following subcutaneous injection, radiolabeled monoclonal antibodies bind efficiently to normal and tumor target cells in the lymph nodes (Project # Z01 C3 08359-03 LTB). This finding prompted us to attempt specific therapy using monoclonal antibody conjugates.

An immunotoxin made from the A-chain of ricin coupled to an anti-mouse MHC antibody has proved to be highly toxic for lymphoid cells and similar results have been found with ricin A-chain conjugated to monoclonal antibodies against subsets of mouse lymphocytes in vitro. We have also been able to augment the effects of these toxins by the action of certain drugs which are known to affect cell biological processes. These studies have led to a new hypothesis for the cell biological pathway (the "neutral bypass") by which toxin molecules enter the cytoplasm from antibody conjugates to kill a cell.

A further type of immunoconjugate for specific cell killing in vivo consists of a radioactive compound chelated to an antibody. We have demonstrated selective ablation of lymph node B lymphocytes in mice injected subcutaneously with an anti-murine B cell antibody labeled with the alpha particle emitter <sup>212</sup>Bismuth. The relative potency of this conjugate for B cells in vivo was 10-fold higher than for T cells taken from the same nodes.

In order to assess the effects of antibodies and antibody conjugates in vivo, we have established two models of lymph node T cell activation. In one, the stimulus is the plant lectin concanavalin A administered into the footpad; the other stimulus is allogeneic cells. Both of these stimuli induce T cells to express receptors for IL-2. The concanavalin A model is susceptible to the inhibitory effects of cyclosporin A whereas the allogeneic model is not.

Inactive in 1988-9 except for writing and publications.

Project Description:Major Findings:

1): Covalent coupling of ricin A-chain to monoclonal antibodies with the SPDP reagent has been obtained using 4 antibodies of different specificities. In each case, there has been little detectable loss of antibody affinity following the coupling reaction. In addition, the ability of the A-chain of ricin to inhibit protein synthesis at the ribosomal level is not significantly impaired.

2): Much of the initial work has been concerned with the monoclonal antibody D3 which recognizes the guinea pig hepatocarcinoma cell line L10. We have been unable to show any specific toxicity due to the D3-Ricin A-chain conjugate against L10 cells in vitro despite the fact that the immunotoxin binds to these cells with an affinity very similar to that of the native antibody and despite the fact that the conjugate is still able to inhibit protein synthesis in an acellular assay. Closer examination of L10 cells showed that they lose some of the antigen recognised by D3 when they are removed from the animal and put into culture. In addition, fluorescence microscopy revealed that these cells are only poorly able to internalize either D3 alone or the D3-ricin A-chain conjugate. The antibody-antigen complex on these cells appear to be capped and then shed into the medium. Such activity could well account for the low reactivity of the D3-ricin A-chain immunotoxin.

3): Immunotoxins having specificities for mouse lymphocyte sub-populations have also been prepared. These reagents have been tested to determine their reactivities to target cells in vitro and have proved to be highly specific and toxic to the appropriate cell type. Drugs which are known to affect intracellular traffic (eg: ammonium chloride, nigericin) have also been used to augment the effects of these immunotoxins in vitro. These findings have led to formulation of the "neutral bypass" hypothesis, i.e., the hypothesis that those few molecules of ricin A which reach the cytoplasm to kill a cell do so via a pathway that bypasses the acidified endocytic vesicles of the "classical" receptor-mediated endocytosis pathway.

Some initial tests have been made in vivo to find out if the immunotoxins are able to kill specific cells or sub-sets of cells in the lymph nodes. We have not yet been able to show substantial cytotoxicity. However, experiments are currently being performed to investigate the kinetics of cell killing in order to optimize this system.

4): We have made an immunoconjugate by chelating the alpha particle emitter <sup>212</sup>Bismuth to a monoclonal antibody through isothiocyanatobenzyl-DTPA. One such conjugate, based on an antibody directed against the murine B lymphocyte determinant LYB8.2, was prepared and used to selectively ablate lymph node B cells in the nodes draining the footpad injection site. At doses which ablated almost all B cells, most of the T cells from the same node remained viable. After treatment, the number of B cells in the node recovered to the pretreatment levels over a period of 48 hours. Following intravenous injection of the same

immunoconjugate, splenic lymphocytes were also ablated although the B cell selectivity was not as high as that seen in the lymph nodes.

5): Immunotoxin experiments demonstrated that lymphocyte killing was much more efficient when the cells were stimulated. In order to optimize immunotoxin killing of lymphocytes in vivo, we have developed two models of in vivo lymphocyte stimulation. In the first, concanavalin A was injected subcutaneously into the footpad. Almost all the lymphocytes from the lymph nodes draining the injection site rapidly expressed the receptor for IL-2 and spontaneously proliferated within 9 hours. In the second model, injection of allogeneic cells caused a smaller number of lymph node lymphocytes to express the IL-2 receptor and to proliferate spontaneously after two to three days. The models induced both B and T cells to express the IL-2 receptor and there was very little difference in receptor expression by either of the two main T cell subsets. Cyclosporin A was able to prevent receptor induction by concanavalin A but not in the allogeneic cell model.

6): Experiments to determine the biological distribution of the uncoupled antibodies following administration via the lymphatic system are described in another report (Project # Z01 CB08359-03 LTB). These experiments must precede the in vivo use of immunoconjugates.

#### Publications:

Weinstein JN. Immunolymphoscintigraphy and other regional applications of monoclonal antibodies. In: Zalutsky M. ed. Antibodies in Radiodiagnosis and Therapy, Boca Raton, FL, CRC Press, 1988;153-167.

Black CDV, Atcher RW, Barbet J, Brechbiel MW, Holton OD III, Hines JJ, Gansow OA, Weinstein JN. Selective ablation of B lymphocytes in vivo by an alpha emitter,  $^{212}\text{Bi}$  bismuth, chelated to a monoclonal antibody. J. Antibody, Immunoconjugates and Radiopharmaceuticals 1988;1:43-53.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08369-06 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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.	1.1	PROFESSIONAL	1.0	OTHER:	0.1
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## CHECK APPROPRIATE BOX(ES)

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

## SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Two areas of research continue to be pursued in the Image Processing Section (Lipkin): development of analytic methods to analyze two dimensional gels (Lemkin), and nucleic acid secondary structure studies (Shapiro). In the first area, development of the GELLAB-II software system was continued, with concentration on increasing the resolution, gel image scanning, quantitation accuracy, capabilities to annotate results and integration of a simplified graphics windowing interface for users, and portability to X-windows based UNIX systems. GELLAB has been used in collaborative studies on axonal proteins, cadmium toxicity to cells, hematological malignancies and long term cell line stability. The RNA secondary structure analysis system has expanded and new algorithms develop to include more functionality for analyzing RNA conformations from perspectives of secondary structure motifs and RNA tertiary structure, and has been made available as a computer workbench to allow a researcher interactively to pursue areas of interest concerning RNA structure. The L11 operon of E. coli has been studied with respect to regulation effects of the L1 protein. Also, the structural effects of the translation efficiency of the lamB gene of E. coli were studied.

The project on development of software for light microscope control has been completed will be documented and made available to the public domain and further work discontinued.



Project Description:Major Findings:

Image analysis. There are three area of research interest in the Image Processing Section (Lipkin): 1) development of methods for analysis two dimensional gels (Lemkin), 2) nucleic acid secondary structure studies (Shapiro) and 3) the hardware and software support (Schultz).

Work on various aspects of the microscope control procedures has been brought to a level where hardware and software specifications are sufficiently detailed to provide users with the requisite platform on which to build various image analysis and other microscope functions to meet individual research needs. Bringing this information into the public domain coincides with the decision to terminate the lease on the Park Building space. Therefore activity in area 3) is brought to an end.

Continued development of GELLAB-II software system with concentration on increasing: resolution, gel image scanning, quantitation accuracy, results annotation capabilities and integration of a user graphics windowing interface. A constant goal during this phase has been to simplify the user's role in performing the exploratory data analysis of a composite 2D gel database. Additional software was written toward achieving this goal. A major part of the effort has been in integrating the UNIX based GELLAB-II software with UNIX workstation based X-windows interactive graphics for portability. A substantial effort was made this year in documenting GELLAB-II and in creating tutorials to facilitate training new users.

Collaborative work has continued with the groups of Dr. P. Sonderegger (U. Zurich) and Dr. E. Lester (U. Tennessee). GELLAB-II has been exported to the University of Zurich. Additional work was required to create the export version of GELLAB and to insure smooth updates of exported versions of the software. Our collaboration with Dr. Sonderegger using GELLAB has helped in finding a secreted axonal protein AXONIN-I. Work is continuing in looking for additional secreted axonal proteins. New collaborations were started within Dr. Sonderegger's Department to analyze 2D gels for cadmium toxicity of epithelial cells and a joint collaboration with Dr. Sonderegger and Drs. A. Amberger and H. Bauer (Salzberg) on long term capillary cerebral epithelial cell lines to aid in understanding the blood-brain barrier. GELLAB-II is included in a course on computer methods in molecular biology (T. Hale, Univ. of Zurich). Work with Dr. Lester of University Tennessee has continued in analyzing sets of lymphocyte gels and long term cell lines from human hematological malignancies to identify potential marker proteins for possible microsequencing. At FCRF, we are using GELLAB-II in a collaboration with Dr. M. Alley to help assess stability of long term cell lines in multiple passages.

Work on nucleic acid structure has continued in a variety of collaborations and directions. The RNA secondary structure analysis system has expanded to include more functionality for analyzing RNA conformations from various perspectives.

This has involved the development of new algorithms to explore secondary structure motifs and RNA tertiary structure. This has made available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA structure in a coherent way. This includes both the use of facilities to explore in detail multiple structures as well as individual RNA structures. This system permits queries of relationships that exist in the RNA secondary structure problem domain involving various software/hardware complexes available at the FCRF. The system is currently being ported to a SUN workstation thus allowing broader access. The system and new algorithms have been used to study the L11 operon of *E. coli* with respect to regulation effects of the L1 protein. Also, the structural effects of the translation efficiency of the lamB gene of *E. coli* was studied.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 08370-06 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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

Other Professional Personnel:David Covell, Ph.D. Expert LTB, NCI  
Peter Greif, M.D. Computer Prog. LTB, NCI

## COOPERATING UNITS (if any)

Hagai Meirovitch, SCRI, Florida State Univ., Tallahassee, FL  
Wilma Olson, Dept. Chemistry, Rutgers Univ., New Brunswick, NJ

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

1.6

## PROFESSIONAL

1.6

## OTHER:

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 novel approach has been taken to the problem of protein folding that examines the complete range of folded topologies accessible in the compact state of globular proteins. The procedure is to generate all conformations, with volume exclusion, upon a lattice in a space restricted to the individual protein's known compact conformational space. This approach affords an enormous reduction in the number of conformations that must be considered for the complete problem. Five proteins have been treated using this method; avian pancreatic polypeptide (36 residues), crambin (46 residues), rubredoxin (52 residues), pancreatic trypsin inhibitor (58 residues), and neurotoxin (62 residues). All conformations generated are evaluated in terms of residue-specific, pairwise contact energies that favor non-bonded, hydrophobic interactions. Native structures for these proteins are always found within the best 3% of all conformers generated. Additional methods are being developed to further narrow the choice of the best among the good conformers. One approach superimposes all atoms onto the lattice, and refines this structure with conventional molecular dynamics and energy minimization. For the test case of one low energy lattice conformation for crambin, an all-atom structure was built that was within 1.46 Å RMS deviation from the native crystal structure. These methods are simple and general and can be used to determine most favorable overall packing arrangements for the folding of specific amino acid sequences within a restricted space.

Project DescriptionMajor Findings:

A principal goal of molecular biology is to understand the bases of molecular and biological recognition. An ultimate goal for theory in this area remains the calculation of favored macromolecular conformations directly from their sequences. Although we occasionally utilize detailed atom-atom calculations, we feel the development of higher order principles of molecular structure is essential if we are to achieve a complete understanding of all of the complexities of biological macromolecules themselves, as well as their interactions with other small molecules, other macromolecules and their assembly into biological structures. This project is progress in that direction.

We have collected statistics on globular proteins from their X-ray structures, counting the amino acid residues that are frequently found near one another in the three dimensional structures. These were obtained in the following way: a lattice model is used in which each residue type has a coordination number. If a specific residue has an incompletely filled coordination shell, then it is assumed to be filled with equivalent water molecules. These contact energies follow intuition, with the most favorably interacting pairs being hydrophobic residues. This type of energy has a direct bearing on folding and substantially reflects the effects of water on folding. There is a segregation of hydrophobic and hydrophilic residues. These values reflect the actual situation inside proteins and provide a tool that can be applied to a variety of problems and, in the problem at hand, can be used to assess the relative overall quality of different conformations.

The present example of generating all possible compact conformations on dense lattices indicates that it should be possible to generate all compact conformations of any small protein, with one lattice point per amino acid. Subsequent addition of the complete atomic details then permits detailed examination of local packing arrangements that favor interactions of side-chains within the protein's interior. By using this relatively coarse-grained approach for examining conformational space and by adding atomic details onto this model, it should become possible to examine the role of amino acid sequence on three-dimensional structure. We have begun additional studies to use simulated annealing to achieve conformational transitions in order to develop approximate methods for larger proteins.

Publications:

Covell DG, Jernigan RL. Lattice Models of Globular Proteins. Correlations Between Sites. Fourth International Conference on Supercomputers, Santa Clara, 1989;II:357-360.

Jernigan RL, Margalit H, Covell DG. Coarse graining conformations: A peptide binding example. In press: Theoretical Biochemistry and Molecular Biology; A Computational Approach, Adenine Press, Schenectedy, New York, 1989.



Jernigan R, Davies D, Scheraga H. Experimental and theoretical protein folding.  
J. Biomol. Str. and Dyn., in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 CB 08371-06 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Conformational Variation 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

## Other Professional Personnel:

Akinori Sarai, Ph.D.	Visiting Associate	LTB, NCI
Bruce Shapiro, Ph.D.	Computer Specialist	IPS, LTB, NCI
Victor Zhurkin, Ph.D.	Visiting Scientist	LTB, NCI

## COOPERATING UNITS (if any)

Dr. Ruth Nussinov, Institute of Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; Dr. Jacob Mazur, Gary W. Smythers, and Dr. David Wang, Program Resources, Inc., Frederick, MD

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

0.9

## PROFESSIONAL:

0.8

## OTHER:

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.)

Conformational analysis of DNA shows that the origin of the B-form double helix can be substantially attributed to the atomic charge pattern in the base pairs. The pattern favors specific helical stacking of the various base pairs. The base pairs alone, without the sugar-phosphate backbone have a strong tendency to be helical. The backbone appears to play a relatively passive role in determining the helix form of DNA. Both electrostatic and van der Waals interactions play a role, but electrostatic interactions are particularly important in A-form DNA.

Sequence studies of bases flanking homooligomer tracts indicate that there are strong biases: usually preferred are bases complementary or identical to the run. A or T runs are preferentially flanked by A's or T's whereas G or C runs are preferentially flanked by G's or C's.

Project Description:Major Findings:

We have studied the sequence dependence of DNA conformational transition between B- and A-forms. The role of intramolecular interactions between base pairs, without backbone, has been examined for the conformational transition between B- and A-form helices. The base pairs themselves usually have intrinsic conformational preferences for B- or A-form. Calculation of all ten possible base steps shows that the base combinations, CC (or GG), GC, AT, and TA, have tendencies to assume A-conformation. Results show that it is particularly easy to slide along the long axis of the base pair for these steps, with AT and CC showing especially flat energies. However, a preference for B- or A-conformation depends on the electrostatic energy parameters, in particular, on dielectric and shielding constants; A conformation is preferred for low dielectric constant or low shielding. Both A and B conformations are stabilized mainly by electrostatic interactions between favorably juxtaposed atomic charges on base pairs. However B conformation generally has more favorable van der Waals interactions than A form. These sequence-dependent conformational preference and environmental effects show substantial agreement with experimental observations.

Also, we have studied the sequence dependence of flexibility and its anisotropy along various conformational variables of DNA base pairs. Our results show the AT base step to be very flexible along twist coordinate. On the other hand, homonucleotide steps, GG(CC) and AA(TT), are among the most rigid base steps. For a roll motion that would correspond to a bend, the TA step is most flexible, while the GG(CC) step is least flexible. The flexibility of roll is quite anisotropic: the ratio of fluctuations toward the major and minor grooves is the largest for the GC step and the smallest for the AA(TT) and CC steps. Propeller twisting of base pairs is quite flexible, especially of A-T base pairs; propeller twist can reach  $19^\circ$  by thermal fluctuation.

Publications:

Sarai A, Mazur J, Nussinov R, Jernigan RL. Origin of DNA helical structure and its sequence dependence. *Biochemistry* 1988;27:8498-8502.

Jernigan RL, Sarai A, Covell DG, Mazur J. Exhaustive enumeration and evaluation of feasible conformations for double helix DNA and for folded proteins. *International Conference on Supercomputers, Boston, 1988;1:197-200.*

Nussinov R, Sarai A, Smythers CW, Jernigan RL. Sequence context of oligomer tracts in eukaryotic DNA: Biological and conformational implications. *J Biomol Str Dyn* 1988;6:543-562.

Mazur J, Sarai A, Jernigan RL. Sequence dependence of the B-A conformational transition of DNA. *Biopolymers*, in press.

Sarai A, Mazur J, Nussinov R, Jernigan RL. Sequence dependence of DNA conformational flexibility. *Biochemistry*, in press.

Nussinov R, Sarai A, Smythers CW, Jernigan RL. Distinct patterns in homooligomer tract sequence context in prokaryotic and eukaryotic DNA. *BBA*, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08374-05 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Membrane Fusion: Structure, Topology and Dynamics: Cell Junctions

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

P. Pinto da Silva, Ph.D.

Chief

LTB

NCI

Others: K. Fujimoto, Ph.D.

Visiting Fellow (to 12/88)

LTB

NCI

## COOPERATING UNITS (if any)

Institute of Experimental Cytology, University of Porto, Portugal (A. Aguas); Dept. of Anatomy, University of Kyoto School of Medicine, Kyoto, Japan (K. Fujimoto)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Membrane Biology Section

## INSTITUTE AND LOCATION

National Cancer Institute, Frederick Cancer Research Facility

## TOTAL MAN-YEARS:

0.4

## PROFESSIONAL:

0.4

## 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.)

We used the acrosome reaction of boar sperm cells to study the dynamics of surface transmembrane glycoproteins (TMG) during secretion. The acrosome reaction is the  $Ca^{2+}$ -dependent fusion of a large cytoplasmic vesicle (the acrosome) with the overlying segment of the plasma membrane (acrosomal cap) that leads to the release of the acrosomal enzymes. We followed the acrosome reaction by freeze-fracture electron microscopy, in particular the topographical rearrangement of a population of acrosomal-cap intramembrane particles that we showed before to correspond to transmembrane lectin (wheat germ agglutinin)-binding proteins (Aguas & Pinto da Silva, *J. Cell. Biol.*, 97:1356). We found that these TMG move in the direction of either one of two opposite poles, proximal and distal, of the cap. This bimodal movement of the TMG reorganizes the acrosomal cap into three domains: the first two, the apical rim and the equator, are membrane domains where the integral proteins are directed to, accumulated, and form small aggregates; the third, comprises the extensive in-between area from where TMG were displaced. The topography of the new membrane domains of the acrosomal cap is now coincident with that of the unaltered domains of the acrosomal membrane underneath. The mirroring of the acrosomal membrane by the plasma membrane is followed by fusion between the two membranes, formation of a labyrinth of tubules, and exocytosis of the acrosomal contents through intertubular fenestrae.

## PROJECT DESCRIPTION

Major Findings:

To fertilize the egg, the sperm cell must penetrate the cell layer (cumulus oophorus) and the glycoprotein coat (zona pellucida) of the oocyte (Bedford & Cooper, 1978; Wassarman, 1987). Removal of these egg vestments is obtained upon activation of lytic enzymes released by the acrosome, a large vesicle of the sperm head (Harrison, 1983; Eddy, 1988). Apposition, fusion and fission of plasma and acrosomal membranes of the sperm cell constitute the acrosomal reaction, the secretory event necessary for fertilization to occur: the phenomenon makes the oocyte's surface accessible to sperm penetration. We used the acrosome reaction in boar sperm to investigate the dynamics of the surface transmembrane glycoproteins during  $\text{Ca}^{2+}$ -triggered secretion. Our previous fracture-label studies showed that transmembrane glycoproteins (TMG) of the sperm head are represented in conventional freeze-fracture replicas by the large, tall intramembrane particles (IMP) seen on plasma-membrane protoplasmic faces (Aguas & Pinto da Silva, 1983). Therefore, we studied the  $\text{Ca}^{2+}$ -induced movement of these IMP to follow the dynamics of TMG during secretion.

The acrosome reaction was induced in spermatozoa according to the method of Green (1978), as adapted for boar sperm by Russel and coworkers (1979). The sperm samples were incubated at  $37^{\circ}\text{C}$  in HBSS containing 2 mM  $\text{CaCl}_2$  and 10  $\mu\text{M}$  A23187 ionophore (stock solution in DMSO). Other samples were used as controls of the calcium effect; they were incubated in the same medium in absence of  $\text{CaCl}_2$  (HBSS + A23187). Sperm aliquots were also incubated in absence of the ionophore (HBSS +  $\text{CaCl}_2$ ). The reaction was arrested at 1, 5, 10 and 60 minutes by glutaraldehyde fixation (1.5% in HBSS, pH 7.4, for 60 min at  $4^{\circ}\text{C}$ ). The percentage of sperm cells that underwent the acrosome reaction was evaluated by Nomarski, phase and bright-field light microscopy. We showed that incubation of boar spermatozoa in 2 mM  $\text{Ca}^{2+}$ , in presence of 10  $\mu\text{M}$  A23187 ionophore, triggers a global displacement of the large IMP of the sperm-head plasma membrane. Our "fracture-label" studies identified these sperm IMP as TMG that bind the lectin wheat-germ agglutinin (WGA) (Aguas & Pinto da Silva, 1983). Therefore, the lateral movement of large IMP reported here reflects translational displacements of TMG. We found that this translational displacement of TMG involves migration over unusually large distances (up to 2  $\mu\text{m}$ ), occurs with 1-5 minutes after  $\text{Ca}^{2+}$  addition to the medium, and ends when the glycoproteins are accumulated at two opposite poles (rim and equator) of the sperm-head surface. As in absence of  $\text{Ca}^{2+}$ , the displacement of IMP does not occur, we conclude that  $\text{Ca}^{2+}$  is associated to the translational displacement of TMG in the area of the sperm-head surface located over the acrosomal vesicle (acrosomal-cap). The new location of TMG leads to the bimodal (transmembrane protein-rich/protein-poor) organization of the entire acrosomal cap into just three large, new domains. The resulting topography of the  $\text{Ca}^{2+}$ -induced domains on the sperm plasma-membrane coincides with that of the stable, structural domains of underlying outer acrosomal membrane.

Our results strengthen the evidence on the importance of WGA-binding glycoproteins of the sperm surface on the modulation of acrosome reaction. Previous studies by Vacquier's group (Lopo & Vacquier, 1980; Podell & Vacquier, 1984; Podell *et al.*, 1984; Trimmer *et al.*, 1985) showed that WGA blocks the influx of  $\text{Ca}^{2+}$  in echinoderm sperm and, in consequence, the acrosome reaction. Biochemical characterization of the WGA receptors of sperm plasma membranes identified

an integral 210 KD glycoprotein (Podell *et al.*, 1984). Antibodies against this WGA-binding glycoprotein inhibit the  $\text{Ca}^{2+}$  influx and the acrosome reaction (Trimmer *et al.*, 1985; Trimmer *et al.*, 1987). Because the bimodal TMG displacement occurs before any membrane fusion events, we postulate that the WGA-binding TMG, on changing their planar topology, may be involved in the formation and/or activation of the sperm-surface  $\text{Ca}^{2+}$ -channels as required for the acrosome reaction to occur. The inhibitory effects of both WGA and specific antibodies on the acrosome reaction would, therefore, be a consequence of the restricted mobility of the TMG upon crosslinking by the ligands.

Publications:

Aguas A, and Pinto da Silva P. Bi-modal re-distribution of surface trans-membrane glycoproteins during  $\text{Ca}^{++}$ -dependent secretion (acrosome reaction) in boar spermatozoa. *J Cell Sci*, In press.

Pinto da Silva P. A non-averaged view of biomembranes: The freeze-fracture image. In Aloia R, Curtain C, and Gordon LM eds. *Advances in Membrane Fluidity. Methods for Studying Membrane Fluidity*. New York: Alan R. Liss, Inc., 1988;1:147-160.

Pinto da Silva P. Geometric topology of membrane fusion: from secretion to intercellular junctions. In: Ohki S, Doyle D, Flanagan TD, and Hui SW eds. *Molecular Mechanisms of Membrane Fusion*. New York: Press, 1988;521-529.

Fujimoto K, and Pinto da Silva P. Apical views of the zonula occludens revealed by freeze-fracture of toad bladder epithelia, Submitted for publication.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08376-05 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Fracture-label: Identification and Partition Transmembrane Proteins

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

P. Pinto da Silva

Chief

LTB NCI

## COOPERATING UNITS (if any)

Institute of General Pathology, Univ. of Rome School of Medicine, Rome, Italy (M.R. Torrissi, A. Pavan, P. Mancini, L. Frati)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Membrane Biology Section

## INSTITUTE AND LOCATION

National Cancer Institute, Frederick Cancer Research Facility

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## 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.)

We used fracture label to examine the partition of the transmembrane proteins that bear CD4 antigens in human lymphocytes. Experiments in both capped and uncapped cells show that, on freeze-fracture, the antigens are preferentially labeled with the inner (protoplasmic) halves of the membrane. This partition illustrates the transmembrane attitude of the antigen molecules and is a probable consequence of interaction of the protein with other components of the membrane or with the cytoskeleton.



## PROJECT DESCRIPTION

Major Findings:

The CD4 antigens expressed on the surface of human T lymphocytes are transmembrane glycoproteins whose sequence and peptide chain topology is now established. The CD4 antigen characterizes a subset of T cells and appears to function as a recognition site for class II HLA antigens and to be a part of the receptor for HIV. We have previously shown that with "Fracture-label" (a method developed in our laboratory) it is possible to study the partition of transmembrane proteins in freeze-fractured cells. In this method, fixed cells in suspension are included into a cross-linked protein matrix, glycerinated, and freeze-fractured by repeated crushing in lipid nitrogen. The fractured specimens are thawed, de-glycerinated and then labeled. We used human peripheral lymphocytes embedded in 30% BSA cross-linked by glutaraldehyde. For our experiments, we used lymphocytes where CD4 antigens remained in its native distribution or were induced to cluster and, eventually, cap by incubation in OKT4 monoclonal antibodies (1 hr at 4°C) followed by incubation with anti-mouse IgG-FITC. The resulting "gel" was sliced, cryoprotected in 30% glycerol and rapidly frozen in LN<sub>2</sub>. The frozen gels were fractured in liquid nitrogen with a pre-cooled glass pestle, and thawed in 1% glutaraldehyde. To analyze the partition of CD4 antigens, we immunolabeled freeze-fractured lymphocytes with monoclonal antibody OKT4 and colloidal gold - protein A. We observed that, whereas labeling over protoplasmic halves of the plasma membrane was moderate, exoplasmic halves remained virtually unlabeled. Next, we analyzed the partition on fracture of CD4 antigens in cells where we induced clustering of the antigens before fixation and fracture. In those specimens, CD4 antigens were also confined to the protoplasmic halves of plasma membranes but with the colloidal gold now clustered. The preferential partition of CD4 antigens with the protoplasmic fracture-face indicates that the transmembrane protein expressing the CD4 antigen, despite its short cytosolic tail, may interact with other membrane components or with components of the cytoskeleton. This behavior is retained even in capped cells. The study of capping of CD4/CD4 antigens is now being continued by fracture-flip methods.

Publications:

Pavan A, Mancini P, Frati L, Torrisi MR, and Pinto da Silva P. Molecular cytochemistry of CD3 and CD4 antigens in human lymphocytes as studied by label-fracture and by fracture-label. *Biochim Biophys Acta* 1989;978:158-168.

Pinto da Silva P. Freeze-fracture images: 1967-1971. In: Hui SW ed. *Freeze-fracture Studies of Membranes*. Boca Raton: CRC Press Inc., In press.

Torrisi MR, Mancini P, and Pinto da Silva P. Cytochemical access to plasma and intracellular membranes of freeze-fractured hepatocytes and salivary gland cells. In: Motta P ed. *Ultrastructural Pathology*. Italy, In press.

Torrisi MR, Pavan A, and Pinto da Silva P. Freeze-fracture cytochemistry of transmembrane proteins in human lymphocytes. *EEO J Immunol Immunopharm* 1988;8:121-123.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 CB 08377-05 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

The Role of Myoglobin in Oxygen Transport

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

David G. Covell, Ph.D.

Expert

LTB, NCI

## COOPERATING UNITS (if any)

r. John Jacquez, Department of Physiology &amp; 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.

0.05

## PROFESSIONAL

0.05

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

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

## SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

We have examined the role of myoglobin to facilitate oxygen diffusion to active mitochondria in skeletal muscle by constructing computer simulation experiments. Steady state mitochondrial oxygen consumption under different conditions of supply  $PO_2$ 's in a system with and without myoglobin were examined for a one-dimensional slab of tissue. Oxygen consumption by mitochondria was saturable with the mitochondria located in bands at uniform intervals throughout the tissue. Under these conditions, myoglobin provides a measurable increase in oxygen transport for supply  $PO_2$ 's below 10 torr and diffusion lengths expected for skeletal muscle fibers. We conclude that only under circumstances where hypoxia lowers  $PO_2$  below 10 torr does myoglobin begin to provide a measurable increase in oxygen delivery to mitochondria.

Project DescriptionMajor Findings:

When examined for a mathematical model that is more consistent with muscle histology, that is, where bands of oxygen consumption are spaced at uniform intervals throughout the tissue, the role of myoglobin becomes clearer. Under these conditions myoglobin provides a measurable increase in oxygen transport for  $PO_2$ 's below 10 torr at the surface and for diffusion lengths expected for skeletal muscle fibers. These results a) illustrate the importance of myoglobin for oxygen delivery to active skeletal muscle during conditions of hypoxia, and b) define conditions where oxygen delivery via free or myoglobin-facilitated diffusion begins to limit the normal function of tissue. This information is important for understanding the role of oxygen supply in the growth and development of normal and tumor tissues and for designing therapeutic strategies for the treatment of tumors and of hypoxia-damaged tissues.

Publications:

Covell DC, Jacquez JA. Does myoglobin contribute significantly to the diffusion of oxygen in red skeletal muscle? *American Journal of Physiology*, 1987; 252 (Regulatory Integrative Comp. Physiol. 21): R341-R-347.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08378-05 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Label-fracture: Topochemistry and Dynamics of Cell Surfaces

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

P. Pinto da Silva, Ph.D. Chief LTB NCI

Others: K. Fujimoto, Ph.D. Visiting Fellow (to 12/88) LTB NCI

P. Pimenta, Ph.D. Visiting Fellow LTB NCI

S. Ru-Long, M.D. Visiting Fellow LTB NCI

## COOPERATING UNITS (if any)

Institute of General Pathology, University of Rome School of Medicine, Rome Italy  
 (M. R. Torrissi, A. Pavan); Dept. Biology, Georgetown University, Washington, DC/USA  
 (D. Nishioka, R.D. Ward)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Membrane Biology Section

## INSTITUTE AND LOCATION

National Cancer Institute, Frederick Cancer Research Facility

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We used label-fracture and replica-staining label-fracture to investigate the distribution and the dynamics of displacement of receptors on cell surfaces. Both methods provide coincident images of the distribution of a surface receptor (labeled by colloidal gold immunocytochemistry) and of the architecture of the membrane interior as exposed by freeze-fracture. With Drs. M. R. Torrissi and A. Pavan, we continued the study of capping by CD3/CD4 and of HLA antigens. Immunogold labeling shows that the process of capping involves initially the migration of all antigen molecules into small clusters which then grow by accretion into longer patches and, finally, a cap. No discernible changes are visible on the fracture faces of capping cells. However, when the aggregation of intramembrane particles is induced, immunogold labeling is confined to the particle aggregates. We proposed that movement and congregation of receptor molecules into a cap is achieved with simultaneous emigration of other transmembrane proteins from the same area, in a process of influx/efflux. In other studies (see Fracture-flip, project #Z01 CB 08387-02), we used label-fracture and fracture-flip to define the surface density and pattern of distribution of lectin receptor sites on the surfaces of the main developmental stages of *Trypanosoma cruzi*. Studies of the topochemistry of sea urchin sperm (S. Ru-Long with Dr. D. Nishioka, Georgetown University) are now in progress to investigate the distribution of specific proteins in several developmental stages of the spermatozoon using monoclonal antibodies/protein 4-colloidal gold.



## PROJECT DESCRIPTION

Major Findings:

To investigate the native distribution and the dynamics of capping of CD3/CD4 and of HLA surface antigens, we used human peripheral lymphocytes isolated by Ficoll/Hypaque density gradient centrifugation. The native distribution of HLA antigens was assessed by treating lymphocytes with anti-HLA A,B,C (class I) or anti-HLA Dr (class II) monoclonal antibodies followed by incubation with anti-mouse IgG-FITC (for CD3/CD4 see "Fracture-label," Z01 CB 08376-04). Clustering and capping of HLA antigens was induced by incubation in anti-HLA solutions (1 hr; 4°C) followed by anti-mouse IgG (FITC) (1 hr at 4°C, wash; then 3 to 20 min at 37°C). All cells were labeled with colloidal gold coated with protein A. The native distribution of the receptors (CD3/CD4 and HLA) appears uniform over the cell body and is, apparently, random. The onset of capping is characterized by a process that involves simultaneous clustering of the antigens, congregation of the clusters into large patches and, eventually, a single cap. No "stray" molecules (an indication of a asynchrony) are observed. Observation of the freeze-fracture morphology of both exoplasmic and protoplasmic faces fails to reveal any aggregation or segregation of particles (intra-membrane particles [IMP] taken to reflect apolar segments of transmembrane proteins). However, when we induced the aggregation of the IMP (by glyceration of unfixed cells) we found that all label [CD3] was confined to the aggregates of IMP. We concluded that the antigens represent components that are visualized in freeze-fracture preparations as IMP, but that their clustering and patching must involve the efflux of other transmembrane proteins away from the same area. Of interest is the fact that all molecules appear to participate in the process, as no isolated molecules are labeled over noncapped areas. This is of interest because it is unlikely that all (or even a majority) of the antigen molecules are crosslinked by the antibody. We are now continuing these investigations using fracture-flip and replica-staining fracture-flip.

Label-fracture studies of the main developmental stages in *Trypanosoma cruzi* using colloidal gold labeled lectins easily establish a cytochemical differentiation between amastigotes, trypomastigotes and epimastigotes. The cytosome (an IMP-free domain) is densely labeled by concanavalin A. We have now started a label-fracture study of sea urchin sperm. Our aim is to establish a cytochemical differentiation of principal developmental stages using specific monoclonal antibodies. Preliminary experiments using wheat germ agglutinin show the presence of a heavily labeled annulus near the top of the head, no labeling at the tip and a relatively uniform distribution of WGA receptor sites over the remaining of the head and the entire length of the tail.

Publications:

Andersson-Forsman C, and Pinto da Silva P. Label-fracture of cell surfaces by replica-staining. *J Histochem Cytochem*, 1988;36:1413-1418.

Kan FWK, and Pinto da Silva P. Colloidal gold label-fracture cytochemistry. In: Hayat MA ed. *Modern Cytochemistry*, In press.

Pavan A, Frati L, Torrisi MR, and Pinto da Silva P. Distribution and dynamics of CD3 and CD4 receptors of human lymphocytes as studied by fracture-label and label-fracture. *Biochem Biophys Acta*, 1988;978:158-168.

Pavan A, Torrisi MR, Frati L, and Pinto da Silva P. The capping of HLA glycoproteins in human lymphocytes as followed by label-fracture. *J Cytochem Histochem*, In press.

Pinto da Silva P. Visual thinking of biological membranes: from freeze-etching to label-fracture. In: Verkleij A ed. *Immunogold Labeling Methods*. Boca Raton: CRC Press, In press, 1988.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08379-05 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Multicompartmental Analysis of Calcium Metabolism

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

David G. Covell, Ph.D.

Expert

LTB, NCI

## COOPERATING UNITS (if any)

Dr. Alfred Yergey, Lab. Theoretical &amp; 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:

0.05

## PROFESSIONAL

0.05

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

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

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

Abnormal calcium metabolism in adolescent children and postmenopausal women can have devastating consequences. The objective of these studies is to elucidate the kinetics of calcium metabolism in normal children and to evaluate disease related changes in calcium metabolism in children and adults. Stable calcium isotopes were administered to children and women of childbearing age and serial samples were obtained for two to four days. Two stable isotopic tracers were used in these studies; one given orally and one given intravenously. The use of two tracers allows direct measurement of several important parameters of calcium metabolism, principally the fraction of calcium absorbed orally and the endogenous fecal excretion. Thermal ionization isotope ratio mass spectrometry was used to measure tracer enrichments in serum, urine, feces and food. The data was used to develop a multicompartmental model of calcium metabolism that better characterized calcium metabolic fluxes between regions of the body.

Project Description:Major Findings:

Significant findings have been made with respect to calcium absorption as a function of age and with respect to estimating the distribution of total body calcium from kinetic measurements. Women beyond the age of forty-five demonstrate a significantly slower rate of absorption of dietary calcium than younger women. Premature infants have a significantly faster rate of absorption of dietary calcium when compared to adolescents. The affect of these findings on total body calcium metabolism have yet to be determined. We hypothesize that gut motility may play an important role in determining the amount of calcium absorbed from dietary sources. In particular, the slower absorption of dietary calcium in older women may signal the beginnings of osteoporosis. The compartmental model developed from our data indicates that the internal distribution of total body calcium can be estimated from measurements of blood calcium and urinary calcium. This approach may serve as a relatively simple method for clinically predicting the onset of disorders of calcium metabolism. Additional data is being collected for further testing of the proposed compartmental model. This research is being conducted in collaboration with Dr. A. Yergey of the Laboratory of Theoretical and Physical Biology. These studies advance the understanding of total body calcium metabolism in adult women and may lead to a method of advance warning for the onset of osteoporosis and improved methods of maintaining calcium balance in premature infants.

The proposed course of this study is to increase the data base used to develop the multicompartamental model describing calcium kinetics. In addition to measuring calcium we will include measurements of hormones (PTH) and vitamin-D. These substances are integral participants in the control of calcium homeostasis and their roles will be included in the control portion of the compartmental model.

Publications:

Hillman L, Tack E, Covell DG, Vieira NE, Yergey AL. Measurement of true calcium absorption in premature infants using intravenous  $^{45}\text{Ca}$  and oral  $^{47}\text{Ca}$ . Pediatric Research, 1988;23:589-594.

Foster DM, Covell DG, Berman M. Applications of a general method for deconvolution using compartmental analysis. Computer Programs in Biology and Medicine, 1988;18(4):32-52.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08330-05 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

Molecular Structure of Animal Viruses and Cells by Computational Analysis

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

Jacob V. Maizel, Jr., Ph.D. Chief, Laboratory of Mathematical Biology, LTB, NCI

## Other Professional Personnel:

Devjani Chatterjee, Ph.D.	Visiting Associate	LTB, NCI
John Owens	Computer Specialist	LTB, NCI
Ruth Nussinov, Ph.D.	Consultant	LTB, NCI
Lewis Lipkin, M.D.	Chief, Image Processing Section	LTB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

4.5

## PROFESSIONAL:

3.5

## OTHER:

1

## CHECK APPROPRIATE BOXES)

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

## SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Viral systems are used to model the complex macromolecular processes and organization of normal, infected and transformed cells. Computers are used to study the nucleic acid and protein sequences that embody the information necessary for these systems to function.

Picornaviruses cause diseases typified by polio, colds, hepatitis, and foot-and-mouth diseases. Gene and protein sequences of these viruses are comparatively studied for relationships among them and to other known and hypothetical proteins. Secondary structures of the RNAs are found to vary in correlation with pathological and sequence differences.

Adenoviruses are studied with a goal to understanding early events in virus replication wherein the cell's metabolism is subverted to viral functions, and late events during which assembly and morphogenesis occurs. Early viral proteins, whose existence was known from biochemical studies, have been analyzed by comparing their sequences to cellular proteins of known function.

Computer analyses of proteins and nucleic acids have been developed and implemented in conjunction with techniques of biochemistry, virology, and electron microscopy on sequences of picornaviruses, adenoviruses, human immunodeficiency viruses. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Computer programs are developed locally and elsewhere for application on Cray XMP, VAX and graphic workstations to perform sequence analysis and structure predictions. Methods to assess the significance of predictions use Monte Carlo simulations, evolutionary comparisons and biochemical data. Roles for genes and proteins are deduced by comparison with databases of sequences of known function and structure.

Project Description:Other Professional Personnel (Continued):

Anne Barber, M.D.	Sr. Staff Fellow	LTB, NCI
Bruce Shapiro, Ph.D.	Computer Specialist	LTB, NCI
Manuel Peitsch, Ph.D.	Visiting Fellow	LTB, NCI
Shu-Yun Le, M.S.	Visiting Fellow	LTB, NCI

Major Findings:

Multiple sequence alignment for the detection of signals: Multiple sequences for related genes provide a powerful data set for detection of structural entities that are involved in recognition by enzymes or regulatory elements, and for understanding common structural motifs. A dynamic program, ANA, for creating alignments of hundreds of sequences, extracting the features and assessing the statistical significance of such alignments has been developed on a Silicon graphics workstation using the C language. Extensions to this program permit rapid assessment of the probability of chance occurrence for a given feature using a method based on binomial probability theory. A similar but non-dynamic program has been developed from the existing FEATUR program in pascal on the VAX computer. Using these programs a number of observations of consensus signals in the vicinity of splice sites, promoter sites and other items contained in the GENBANK "feature" table have been compiled. Analogous programs have been developed for use with amino acid sequences. They are used to find common structural domains between HIV proteins and known families of enzymes such as G-proteins and reverse transcriptases and structural proteins, other viral sequences and potential antigenic sites.

Similarity studies: Sequence comparison is one of the most powerful techniques for understanding the organization and function of biological systems. Some sequencing laboratories feel that with the growing automation of the sequence determination process, the limiting step will be analysis of the sequence data by computer unless superior methods are available. There will be a continuing need for methods to compare sequences both more easily and more thoroughly. Knowledge bases of experience with the meaning and interpretation of results will also be needed. A variety of methods are used in the laboratory. One of the most powerful of these uses the Cray XMP to search for regions of local homology with the thorough program, SEQHP, or the more rapid SEQFP/SEQFT programs of Kanehisa, and then to uses the SEQDP program to assess the non-randomness of the matches. These have now been combined and modified to produce output suitable for direct input into relational database programs such as FRAMIS or INGRES. Low homologies can not be related unambiguously to common evolutionary background, and rather may reflect common structural motifs, or coincidence. Instances of relationships that are obvious in 3-D structure but are difficult by homology (e.g. the two halves of the enzyme rhodanese), may serve as test cases for methods to assess significance. Instances continue to arise where the full dynamic programs and fast hexical based methods differ significantly in results.

An extension of this concept is to use similarities between new sequences and known crystallographic or other 3-D structural data to permit the generation of a molecular model for a protein that can predict and be challenged by experimental data. Plausible models have been generated for perforin, apo-lipoproteins, portions of reverse trans? ??????ase and portion of CD-4 antigens using similarity search (DFASTP, SEQFDP, SEQHDP) molecular mechanical (CHARRM, AMBER) and molecular graphics programs (MOGLI, MIDAS, GEMM). In some cases these results are stimulating new insight into the functions of proteins derived from gene sequencing.

Detection of potential RNA secondary structure: RNA and DNA structure are of increasing concern as the innate catalytic activity, and other functional properties of polynucleotides are being recognized and more experience with protein-nucleic acid interactions is accumulated. Evidence from simple systems suggest that there is a hierarchical precedence to the determination of single-stranded polynucleotide structure in the order that primary sequence determines regions of helical base pairing and stacking that pack into 3-dimensional structures. Thus it is necessary to make reasonable predictions of the secondary structure in preparation to predicting the full structure. Methods have been developed along two lines to assess the secondary structure-forming potential of RNA sequences. In one case alternate structures are explored either by examining sub-optimally folded structures of nearly the same stability as the best ones, and in another method the non-randomness of the structures are examined by comparing the natural sequence with those of the same composition but of varying degrees of random order. Both methods yield agreeable results with experimental data, when it is available. A third method utilizes comparison of structures that appear to be conserved across phylogenetic or mutational challenge. This latter method is widely accepted but has never been assessed by statistical tests. Using all these methods oncornavirus and HIV-related RNA's are being examined.

The method has been improved in efficiency up to 100-fold by an extensive study to replace the time-consuming Monte Carlo procedure with a lookup table using parameters derived from the base composition. In future it will be possible to extend the method to large numbers of sequences, and to provide the capability to use smaller computers for selected sequences.

Regions of significantly stable structures are found to correlate with the tat-regulating region already shown by others, the rev-responsive region (Melvin, Cullen, Le, Maizel) and the frame-shift sites in the gag-pol and other regions of retroviruses. As a trial the entire genome of HIV was folded on a Cray 2 computer at NSAS-Ames through a grant of time. It took approximately 6 hrs as predicted from results with smaller sequences on our Cray XMP/24. This required nearly 150 million words compared to the XMP/24's 4 million words. Predicted localized structures were compared with the global structure and astonishingly it was noted the tat and RRE regions had persisted even though the presence of the entire sequence offered vast possibilities for alternate folding. These observations should be extended. Other extensions of this folding methods have been tested with a large set of more than 100 t-RNA's. Typically a single method will predict only a mini????? of them as being "clover-leaf" morphology, although it is commonly believed to be the universal

structure. Using a method in which the rules are fluctuated to simulate typical experimental results it has been possible to show that the same algorithm can predict more than 80% correctly. This method also will be extended to study other structures. This method finds regions of both greater and less than random stability in HIV sequences. Regions of significant non-stability, or open character, are correlated with the hyper-variable regions of the envelope glycoprotein gene.

#### Publications:

Chen J, Barber AM, Pedersen J, Brandt-Rauf PW, Carucci J, Murphy RB, Pincus MR. Comparative x-ray crystallographic evidence for a beta-bend conformation as the active structure for peptide T in T4 receptor recognition. *J Protein Chem*, in press.

Hausheer F, Barnett G, Colvin M, Maizel JV. Analysis and prospective design of drugs for the treatment of cancer and AIDS by supercomputer. Presentation at ICS 1988 Third Intl Conference on Supercomputing and Second World Supercomputing Exhibition. Boston: Intl Conference on Supercomputing, 1988;201-206.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08381-06 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Computer Aided Two-Dimensional Electrophoretic Gel Analysis (GELLAB)

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

Peter F. Lemkin, Ph.D. Computer Specialist IPS, LTB, NCI

## Other Professional Personnel:

Lewis L. Lipkin, M.D. Chief, Image Processing Sect. IPS, LTB, NCI

## COOPERATING UNITS (if any)

Dr. E. Lester (Univ. Tenn. Ctr. Health Serv.); Dr. P. Sonderegger, T. Hale (Biochem. Inst. Univ. Zurich); Dr. M. Alley (NCI/FCRF); Dr. H. Bauer, Dr. A. Amberger, (Austrian Acad. Wissurschufren, Salzberg); Dr. T. Krekling (Agr. Univ. Norway);  
 Mr. J. Ashmore (PRT/FCRF); Mr. V. Kasprzak (PRT/FCRF).

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Image Processing Section

## INSTITUTE AND LOCATION

NCT/Frederick Cancer Research Facility, Frederick, MD 21701

## TOTAL MAN-YEARS

## PROFESSIONAL

## OTHER

2.6

1.4

1.2

## CHECK APPROPRIATE BOX(ES)

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

B

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

GELLAB is an exploratory data analysis system for the analysis of sets of 2D electrophoretic gel images. GELLAB incorporates sophisticated subsystems for image acquisition and processing, database manipulation and graphics as well as statistical analysis. It has been applied to a variety of experimental systems in which quantitative and qualitative changes in one or more proteins among hundreds or thousands of unaltered proteins is the basic analytic problem. Keeping track of changes detected using these methods is also a major attribute of the system. A composite gel database may be "viewed" under different exploratory data analysis conditions and statistical differences and subtle patterns elucidated from "slices" of an effectively 3D database. Results can be presented in a variety of tables, plots or derived images. The computer workstation based system, GELLAB-II, now runs under UNIX and uses X-windows for portable interactive graphics.

Substantive applications include: analyzing sets of lymphocyte gels and long term cell lines from human hematological malignancies to identify potential marker proteins for possible microsequencing using a gas-phase sequenator (Lester); axonal proteins synthesized during axonal regeneration (Sonderegger); cadmium toxicity of epithelial cells (Sonderegger and Hunziker); cerebral endothelial cell morphology control (Bauer and Amberger); assess stability of long term cell lines (Alley).

Project Description:Major Findings:

Stabilization of GELLAB to run on workstations:

Continued work on stabilizing and converting GELLAB-I to GELLAB-II (GELLAB-I had been converted from SAIL to C in GELLAB-II using the PSAIL translator (2)).

which has been integrated with more of the programs. Integration of DataCopy high resolution CCD camera for scanning gels at higher resolution. Increased size of images in image processing and database software so GELLAB-II can handle these higher resolution gels. A program was developed for general image conversion so that images from any scanner could be converted to the format required by GELLAB-II - facilitating use of GELLAB for use by other laboratories which do not have our scanner.

A 2D gel annotation database facility was added to GELLAB-II. This interactive program can be used to both define and search spot annotation data which may be both textual and computational. It will be used both for record keeping of data from the current experiment as well as information about specific proteins from other laboratories.

Additional GELLAB C-code library procedures were written to support GELLAB programs. This includes a GELLAB-II X-windows library which helps enforce a consistent windowing user interface making it easier to learn GELLAB.

The 25+ GELLAB-II programs (about 125,000 lines of code) are in final stages of completion in terms of fine tuning to simplify the user interface. The composite gel database program CGELP2 has added functionality to integrate it better with many of the other programs so that generation of derived gel images is smoother. Additional effort was made in simplifying the code for eventual maintenance by others. Other changes were enhancements, such as the protein "expression profile" search, involve new gel database search techniques. In preparation for the sharing of 2D gel databases between different laboratories, extensions were made to incorporate "foreign" database spot numberings and annotation information.

As increased 2D gel image resolution is required with recent improvements in 2D gel production (over 2000 spots/gel on standard size gels), our CCD camera can capture this higher resolution. Major changes in many modules of GELLAB-II were made to handle this increased resolution for variable size images.

Additional work was done in optimizing the spot quantitation program so as to minimize computation times for increased resolution. This is important as computation times increase with the square of the image size.

In order to maximize the interfascibility of GELLAB-II with primary data from other 2D gel systems (both academic and commercial systems), some changes were made to the fundamental data structures to allow us to input paired spot CGELP2

data from non-GELLAB sources. This would also allow us to use the statistics and search record-keeping part of GELLAB on other types of biomedical data.

One of our goals is to be able to use digital 2D gel data from other laboratories and analyze it using GELLAB. The PPXCVT program for image conversion of other laboratory formats to our standard format has been completed and is able to convert a variety of 2D gel image formats.

GELLAB-II now has more of the line graphics used in GELLAB-I working. This entailed completely rewriting a DECsystem-10 OMNIGRAPH graphics subsystem. The GELLAB-II program PLOTN is able to replot graphics files produced by the various GELLAB-II programs as either Tektronix-4010 or PostScript - the latter which may be used to get hardcopy laser printer plots.

To facilitate learning GELLAB using the exported software in other laboratories, demonstration program scripts and tutorials were developed for new users. This is being iterated and optimized as we gain experience with what "information" is missing when learning the system outside of our lab.

A book on GELLAB-II is in final stages of preparation. It is intended to be used primarily as a reference for the GELLAB-II system. As such, it contains material which includes a general introduction to gel analysis methods, tutorials on introductory and advanced methods for computer assisted analysis of 2D gels, and a discussion of exploratory data analysis techniques as applied to gel analysis.

#### A Portable Picture System - Xpix:

Work has continued on Xpix, a portable menu driven image processing system for X-windows which currently runs on SUN, microVAX, etc. workstations under UNIX. Xpix is used with GELLAB-II to interactively view, enhance, process, etc. gel images. Xpix has additional capability which makes it suitable for analyzing 1D gels as well as other types of images. It was designed so that new functionality can be added through external UNIX programs rather than in making time consuming and more difficult changes to the program itself. Additional functionality has been added to Xpix to make it more powerful as well as easier to use. It has been rewritten since last year so as to take advantage of the GELLAB X-window library. This library was then used for adding a consistent windowing image interface to other GELLAB programs.

#### Proposal for digital data interchange for Portable 2D gel databases:

During the 2D Gel Electrophoresis meeting in Vienna, Austria November 8-11, 1988 several of the laboratories interested in having portable gel databases formed a group to try and work out a portable file interchange mechanism to exchange 2D gel data between laboratories. I was selected as moderator and have been working on integrating suggestions to try and come up with a scheme agreeable to most of the groups. The goal is to be able to transfer digital 2D gel data.



### Collaborations:

Collaborative work with Dr. Peter Sonderegger at the Biochemistry Institute, University of Zurich has resulted in finding a secreted axonal protein AXONIN-I (4). Work is continuing in looking for additional secreted axonal proteins. The GELLAB-II system which was exported to the Biochemistry Institute at the University of Zurich. GELLAB-II is included in a course on computer methods in molecular biology (Terry Hale). New collaborations were started with Dr. Sonderegger's Department to use analyze gels for cadmium toxicity of epithelial cells (Dr. Hunziker). While in Zurich in November 1988 and May 1989 I met with Mr. Terry Hale, of the Biochemistry Institute to discuss GELLAB-II installation, and its use. In the May visit, I collaborated with members of Dr. Hunziker's group on using GELLAB on their gels. In a joint collaboration with Dr. Sonderegger, I met with Dr. Albert Amberger from Dr. Bauer's lab (Salzberg) to discuss the use of GELLAB on gels of their long term capillary cerebral epithelial cell lines to aid in understanding the blood-brain barrier.

Additional collaborative work with Dr. Eric Lester at University Tenn. Center for Health Services on labeled proteins from human hematological malignancies, including long term cell lines. Work has continued on the 2D gel adult human leukemia and lymphocyte database. Several proteins selected from this database with the help of GELLAB were microsequenced. Some of this data was incorporated in the new annotation database subsystem being developed in GELLAB.

We are exploring with Dr. Michael Alley of NCI-PDR/FCRF the feasibility of using GELLAB-II at FCRF in part of his program. We are evaluating gels from a A549 lung adenocarcinoma TC cell line to assess the level of stability of proteins expressed at multiple passages.

We are exploring with Dr. Trygrv Krekling (Agr. Univ. Norway) on exporting GELLAB-II and collaborating on future enhancements.

Mr. Robert Ashmore (PRI/FCRF) has been collaborating in the conversion of some of the GELLAB-I programs to GELLAB-II. He has also contributed in developing the X-windows interface used in the GELLAB-II programs and in extensions to the Xpix program.

Mr. Voitek Kasprzak (PRI/FCRF) has extended the COMTAL/PDP11-44 image processing system to be accessible from Ethernet so as to be usable as another image processing resource in our laboratory.

GELLAB-II has been exported to the University of Zurich (Sonderegger and Hale) which is our Beta export test site. Additional changes to facilitate continued collaboration and exporting of enhanced versions of GELLAB has been actively pursued.

Much of the time this past year was spent extending and fine tuning GELLAB-II software to take advantage of the UNIX environment. This included new software to control a high resolution CCD camera, enhancements of the gel quantitative and annotation database subsystem, and user windowing interface enhancements.

Publications, lectures and abstracts:

## Publications:

Lemkin PF. GELLAB-II, A workstation based 2D electrophoresis gel analysis system. In: Endler T, Hanash S. (Eds) 2D Electrophoresis. Vienna Austria, November 8-11, 1988. VCH Press: W.Germany, 1989: 5.

Lemkin PF. PSAIL: A portable SAIL to C compiler - Description and tutorial. SIGPLAN Notices, 1988;23(10):149-171.

Lemkin PF, Lester EP. Database and search techniques for 2D gel protein data: A comparison of paradigms for exploratory data analysis and prospects for biological modeling. Electrophoresis, 1989;10(2):122-140.

Stoeckli ET, Lemkin PF, Kuhn TB, Ruegg MA, Heller M, Sonderegger P. Axonally secreted proteins: I. Identification of proteins secreted from axons of embryonic dorsal root ganglia neurons. EMBO J, 1989;180:249-258.

Kasprzak V, Lemkin P, Shapiro B. Short manual for the PDP11/44 - COMTAL service program. IPS, LMMB, NCI/FCRF. 1989;16.

## Books:

Lemkin PF. The GELLAB-II 2D gel analysis system. 1989;557. In preparation.

## Lectures:

Title: GELLAB-II, A workstation based 2D electrophoresis gel analysis system, "2D Electrophoresis Conference", Vienna, Austria, November 8-11, 1988.

Title: 2D Gel Database Analysis Using GELLAB-II. FEBS-IUB-Danish Biotechnology program. Advanced lecture-practical course: "Protein Databases Based on the Analysis of Two-Dimensional Gels". May 8-10, 1989, Institute of Medical Biochemistry and Aarhus University, Bioregulation Research Centre, Aarhus, Denmark.

Title: 2D Gel Database Analysis Using GELLAB-II. Guest lecture, University Zurich, Dept. Biochemistry, November 123, 1988. Collaborate with members of department on installation and use of GELLAB-II in their laboratory.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08382-06 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

System Software for 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:

Hanah Margalit, Ph.D.

Visiting Fellow

LTB, NCI

Kathleen Currey, M.D.

Guest Researcher

LTB, NCI

Ruth Nussinov, Ph.D.

Consultant

LTB, NCI

Lewis L. Lipkin, M.D.

Chief, Image Processing Section

LTB, NCI

Jacob V. Maizel, Jr., Ph.D.

Chief, Lab. of Mathematical Biology

LTB, NCI

## COOPERATING UNITS (if any)

Amos Oppenheim, Ph.D. (Hebrew Univ., Israel) Sarah Lesner (Applications Analyst, PRI) Wojciek Kasprzak (PRI)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Image Processing Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

## TOTAL MAN-YEARS.

2.7

## PROFESSIONAL:

2.0

## OTHER:

.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.)

Work on nucleic acid structure has continued in a variety of collaborations and directions. The RNA secondary structure analysis system has expanded to include more functionality for analyzing RNA conformations from various perspectives. This has involved the development of new algorithms to explore secondary structure motifs and RNA tertiary structure. This has made available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA structure in a coherent way. This includes both the use of facilities to explore in detail multiple structures as well as individual RNA structures. This system permits queries of relationships that exist in the RNA secondary structure problem domain involving various software/hardware complexes available at the FCRF. The system is currently being ported to a SUN workstation thus allowing broader access.

Algorithms have been developed that involve RNA secondary structure analysis specifically the measurement and visualization of structural similarity from global and contextual viewpoints. A very fast algorithm has been developed to compare RNA structures using the concept of levels of abstraction. This permits the definition and searching of structures from loose to a more restricted specifications. A research effort has also begun using the system examining the potential effects of tertiary RNA interactions such as loop-loop bindings.

The system and new algorithms have been used to study the L11 operon of *E. coli* with respect to regulation effects of the L1 protein. Also, the structural effects of the translation efficiency of the lamB gene of *E. coli* was studied.

Work has begun using the structure analysis system to study the structural aspects of polio. Initial observations indicate that secondary and possibly tertiary structural components may correlate with ribosomal binding sites.

Project Description:Major Findings:

A highly integrated system for the analysis of RNA secondary structure has been developed on a SYMBOLICS 3675 computer. One of the objects of this research is to make available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA secondary structure in a coherent way. This includes both the use of facilities to explore in detail multiple structures as well as individual RNA structures. This system is forming the basis for an expert system which is permitting intelligent queries of relationships that exist in the RNA secondary structure problem domain involving various software/hardware complexes available at the Frederick Cancer Research Facility. The system currently has a large number of functions that permit the use of algorithms that reside on different nodes on the FCRF network. This includes the SUN'S, and VAXES and very shortly the CRAY XMP (Wojciech Kasprzak, PRI). These algorithms are invoked from the SYMBOLICS utilizing one common mouse and window system that reduces the users need to know the various software/hardware complexes. The user has the interactive capability to fold literally hundreds of structures and to cluster these structures to determine which are similar and which are not as well as which substructures are similar and which are not (see 1,2,5). The ability also exists to search for specific structural elements that are a function of global or semi-global structure, base pairing, local energies as well as sequence. The ability exists to graphically display structures that are generated. One may then interact with the display to get at various local structural elements.

The structure may be labeled in different ways so that the important area of current interest may be viewed. Structures may be compared analytically, for example using a Boltzman distribution as well as visually. The system is in the process of being ported to sun workstations (Sarah Leshner, PRI) thus allowing broader access to the research community.

New algorithms and methodologies have been developed that permit the clustering, searching for, and examining in detail of multiple RNA secondary structures from a structural as well as sequence standpoint. The object is to find structural similarities that could be correlated with functional sites. This includes the examination of how mutations affect structure and function in RNA. Examination of the regulation effects of L1 protein and L11 gene of E. coli have been examined in detail using the system with its new set of algorithms (with Margalit, Maizel-LMB, Oppenheim-Hebrew University) (see 2). Mutations may be generated that preserve the amino acid coding sequence and then these structures are then compared to determine which ones are similar to the wild type or other alternate structures. These in turn suggest mutational experiments to perform in the laboratory to determine how the mutants perform in comparison with the predicted models. Comparative studies have been completed with structures published in the literature e.g. L1 and L11. Examination of the L1/L11 data shows some good correlations to the published experimental results. The lamB gene of E. coli have been examined also in regard to how structure effects



translation (see 2). Specifically the local structure surrounding the Shine-Dalgarno region of the Lamb gene was compared using computer generated mutations some of which were compared with experimental published work and suggests other compensatory mutations that should respond like the wild-type.

Work has been pursued involving computer experiments to determine how RNA conformational energies are related to sequence composition. An algorithm has been developed that can determine what the average energy would be for a given sequence composition. This is being utilized to determine the significance of a folded RNA molecule compared to what would normally be expected given its base composition (with Chen, Le, Currey and Maizel-LMB) (see 3).

Work has been pursued involving the determination of a RNA secondary structure consensus. An algorithm has been developed that allows one to determine the structurally significant conformations from a group of conformations thus forming a consensus (with Le, Owens, Nussinov, Maizel) (see 4). The object of this algorithm is to determine what structures within a given sequence are most stable under the influences of folding and to extract those structures to form a consensus structure. The consensus may also be determined by looking at preserved structures generated from suboptimal foldings.

A phase of the work utilizing a new tree comparison algorithm to do flexible and accurate comparisons of RNA secondary structures has been completed (see 2,5). This included the ability to determine precisely where differences reside between to structures and to place a measure on these differences (with Zhang and Shasha-Courant Institute). This is analogous to the sequence homology algorithms commonly used but here structures are being compared. The work is evolved out of a lecture I gave to a graduate course established at the Courant Institute at NYU with participation of various NIH and non-NIH lecturers. The algorithm permits several levels of abstraction in comparing RNA structures. The structures may be compared based upon a loose morphologic description of their shape. A more refined comparison is possible allowing for the sizes of the individual structures to be included. Ultimately sequence information may be included. The algorithm mentioned here has been included in the above described system to get an even better handle on comparative structures. The algorithm was utilized in the research done in (2). The algorithm appears to be useful as an aid in phylogenetic studies of RNA secondary structures.

Work has begun in collaboration with Dr. Kathleen Currey of University of Maryland Medical School on the structural effects of virulent and non-virulent polio strains I, II and III. This has led to the development of new algorithms to permit consideration of RNA tertiary structure, specifically loop-loop interactions which includes the possibility of pseudo-knots, in regard to polio's functional sites. Experimental mutational data is being included (Trono et. al., 1989) to better relate the structural aspects to functional regions of the 5' noncoding regions. Initial observations suggest that secondary structure and possibly tertiary structure in the form of neighboring stems with loop-loop interactions may correlate with ribosomal binding sites.

Publications:

Shapiro BA. An algorithm for comparing multiple RNA secondary structures. CABIOS, 1988;4(3):387-393.

Margalit H, Shapiro BA, Oppenheim AB, Maizel JV Jr. Detection of common motifs in RNA secondary structures. Nucleic Acids Res., in press.

Chen J-H, Le S-Y, Shapiro B, Currey KM, Maizel JV. A computational procedure for assessing the significance of RNA secondary structure. CABIOS, in press.

Le S-Y, Owens J, Nussinov R, Chen J-H, Shapiro B, Maizel JV. RNA secondary structures: comparison and determination of frequently recurring substructures. CABIOS, in press.

Shapiro BA, Zhang K. Comparing multiple RNA secondary structures using tree comparisons. Submitted.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08385-03 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

DNA Conformations in Control Regions

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

Robert L. Jennigan, Ph.D.,	Theoretical Physical Chemist	LTB, NCI
Hanah Margalit, Ph.D.,	Visiting Fellow	LTB, NCI
Bruce A. Shapiro, Ph.D.,	Computer Specialist	LTB, NCI
John Owens, Ph.D.,	Microbiologist	LTB, NCI
Ruth Nussinov, Ph.D.	Consultant	LTB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- (a) Human subjects       (b) Human tissues       (c) Neither      3
- (a1) Minors
- (a2) Interviews

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

Extracting molecular details of the mechanism of promoter function in transcription is a subject of broad interest. We have chosen to look at one of the simplest aspects of this process, namely, the strength of DNA double helix strand pairing in the promoter region. Specifically, the stability of the DNA double helix was determined in the vicinity of the promoter by computing the free energy for strand separation as a function of dinucleotide free energy values taken from the calorimetric measurements of Breslauer et al. The stability of 168 E. coli promoter regions was studied within a window of +/- 250 nucleotides on either side of the transcription start sites. We found that for this set of promoters the -10 region was significantly the least stable. There is no correlation between the free energies and the rates of RNA polymerase-promoter open complex formation measured for 25 promoters. We also compare the free energies of 121 promoter mutations across the -35 and -10 consensus regions with the free energies of the corresponding wild type sequences. These pairwise mutant-wild type comparisons provide a particularly good test since the examined sequences differ only in one nucleotide so that all other sequence dependent effects are fixed. About 80% of the mutations in the -10 region that show increased/reduced promoter activity are less/more stable than the wild types. The observed high free energy peak, as well as the mutation data, strongly support the conjecture that the instability, or melting properties, of the -10 region plays a significant role in promoter function.

Project Description:Major Findings:

For a collection of 168 promoters we showed that the -10 region is the least stable paired double helix, within a 500 nucleotide span around the transcription initiation site. We revised our database to include only promoter sequences for which the transcription start positions have been identified experimentally. The present database of 168 E.coli promoters is based on the recent compilation of promoter sequences (Harley and Reynolds, 1987) and on GENBANK release 52. The peak in frequency of TA (the least stable dinucleotide) in the -10 region might suggest that one of its roles is in facilitating the "melting in" step in the open complex formation.

In a data base of 121 up and down promoter mutations, about 80% of the mutations in the -10 region that show increased/ reduced promoter activity are less/more stable than the wild types. There is no correlation between the type of mutation in the -35 region and the direction of free energy change. Also, the -35 region is not so relatively unstable.

The mutation analysis points to a possible dependence of promoter activity on the -10 region helix stability. A possible mechanism that could cause such a dependence is the following:

- 1) The enzyme interacts with the -35 region to form the closed complex.
- 2) The -10 region double helix is opened by thermal fluctuations.
- 3) The enzyme interacts with the appropriate strand in the -10 region to form the open complex.

A kinetic formulation for this process, shows a direct relationship between the free energy and the rate of open complex formation. However, we did not find a linear correlation between the free energies and the rates of open complex formation (available for 25 promoters), implying that the stability of the DNA is not the only factor that determines the rate of the process. The last step might be rate limiting and is probably sequence dependent as well.

The kinetic analysis compares activities of different genes, and the variation among them is probably a result of various sequences affecting the different steps that lead to an open complex formation. In the mutation analysis the activities of a wild type and a mutant of the same gene are compared. The only difference between the two promoters is a single point mutation. All the sequence dependent factors affecting transcription rate are identical except the one at the position of the mutation. The strong correlation between the type of mutation and the direction of free energy change in the -10 region indicates that the ease of melting directly affects the transcription rate.

PUBLICATIONS:

Margalit H, Shapiro BA, Nussinov R, Owens J, Jernigan RL. Helix stability in prokaryotic promoter regions. *Biochemistry* 1988;27:5179-5188.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08386-03 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Beta (1-4) Galactosyltransferase Topology and I's Gene Structure

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

Pradman K. Qasba, Ph.D.

Research Chemist

LTB, NCI

## Other Professional Personnel:

Arni Masibay, Ph.D.

IRTA Fellow

LTB, NCI

Elizabeth Boeggeman, Ph.D.

IRTA Fellow

LTB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL

1.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

 (a) Human subjects (b) Human tissues (c) Neither (a1) Minors

B

 (a2) Interviews

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

To find out the differences between trans Golgi and the cell-surface beta (1-4) galactosyltransferase (GT) enzyme whose synthesis are differentially regulated we continued this year the studies on the primary structure of GT in relation to its gene structure. The 1st exon of GT gene codes for the first 141 residues of the 401 residue long precursor GT protein. 72-residue-NH<sub>2</sub>-terminal peptide of the 141 residue domain, coded by the 1st Exon, contains the membrane anchoring domain and is cleaved to generate the soluble and secreted form of GT. The 1st exon is separated from the second exon by about 12Kb intervening sequence. The amino-terminal end sequence of the low molecular form of secreted and soluble form of the protein, which is enzymatically active, corresponds to the nucleotide sequence of the second exon. We also show that a) there are tissue specific variations in the size of 3'-noncoding sequence of GT mRNAs which are generated by utilizing other potential polyadenylation signals than the canonical sequence. In the lactating mammary gland of various species there is, besides the major 4.2Kb mRNA, a 2.2Kb mRNA which is shorter at the 3'-noncoding region. In addition there are multiple poly(A)<sup>+</sup> RNAs which are devoid of the 3'-end sequence coding for the COOH-terminal end of the enzyme. Our results suggest the possibility that the surface and Golgi GT might be generated by alternate splicing or from second copy of gene or both.

Project Description:Major Findings:Gene Structure of Beta (1-4) Galactosyltransferase:

Overlapping bovine Beta (1-4) Galactosyltransferase genomic clones were isolated from a bovine genomic library. Results of restriction analysis and arrangement of the cDNA sequence in the genome showed that: 1) There are 5 introns and 6 exons in the GT gene. 2) First exon codes for the first 141 residues of the precursor GT protein which has two in-frame methionines 18 residues apart. Out of this 141 residue region, the 72-residue NH<sub>2</sub>-terminal peptide which also contains the membrane anchoring domain, is missing from the secreted and soluble form of the enzyme. 3) There is no intervening sequence in the 2.7Kb long 3'-noncoding sequence. The 6th exon codes for the 41 residue long COOH-terminal portion of GT and contains 2.7Kb 3'-noncoding sequence. 4) The gene sequence coding for residue 142 to 361 of the GT protein contains 4 exons. 5) Sequence analysis of several cDNA clones have shown that some mRNAs contain only 4th exon GT sequence or are missing 5th exon sequence. 6) Several bovine Alu-equivalent sequences are present in the intervening sequences and in the 3'-noncoding sequence.

Northern Blot Analysis of the RNA:

Northern blot analysis show that in addition to the major transcript of 4.1Kb for GT observed in bovine, rat and mouse tissue, there is a minor transcript of 2.1Kb that is detected in a tissue specific (mammary gland) but not in a species specific manner. Hybridization of the RNA blots with the 3'-end probe derived from the clones corresponding to the 4.1Kb mRNA shows the shorter mRNA (2.1Kb) lacks this region. Sequence analysis of several cDNA clones showed that several canonical and potential polyadenylation sites (ATTAAA or TATAAA) present in the 3'-noncoding region are utilized for polyadenylation and account for the shorter transcripts. Some transcripts are generated by utilizing the polyadenylation sites (AATAAA or ATAAAA) present in the intervening sequences and thus lack sequences of remaining exons. These results suggest that complex transcripts are generated from GT gene.

A paper describing these results is in preparation.

Publications:

Masibay AS, Qasba PK. The complex alternative processing of the Beta (1-4) galactosyltransferase precursor mRNA. The J Cell Biology Abs 1989;4842:850a.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08387-02 ITB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

## Fracture-flip: Nanoanatomy of Cell Surfaces

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

P. Pinto da Silva, Ph.D. Chief LTB NCI

Others: K. Fujimoto, Ph.D. Visiting Associate (to 12/88) LTB NCI

P. F. Pimenta, Ph.D. Visiting Fellow LTB NCI

S. Ru-Long, M.D. Visiting Fellow LTB NCI

Cooperating Units 1. Inst. General Pathol., Univ. Rome Sch. Med., Rome, Italy (M.R. Torrisi, A. Pavan). 2. Lab. Parasitic Diseases, NIH/NIAID, Bethesda (R.P. da Silva,

D. Sachs, A. Sher). 3. Inst. Biophysics, Fed. Univ. of Rio de Janeiro, Brazil (W. de Souza, T. Souto-Padron). 4. Dept. Anatomy, Karolinska Inst., Stockholm, Sweden (G. Andersson-Forsman). 5. Dept. Anatomy, Univ. Kyoto Sch. Med., Kyoto, Japan (K. Fujimoto). 6. Dept. Biology, Georgetown Univ., Wash., DC (D.

LAB/RANCH Nishioka, R.D. Ward). 7. Center for Exptl. Cytol., Univ. Oporto, Oporto, Laboratory of Mathematical Biology Portugal (A. Aguas, M. Teixeira da Silva)

## SECTION

Membrane Biology Section

## INSTITUTE AND LOCATION

National Cancer Institute, Frederick Cancer Research Facility

## TOTAL MAN-YEARS:

1.8

## PROFESSIONAL:

1.8

## 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.)

We continued the development and application of fracture-flip, a method that we discovered last year. This new method provides macromolecular resolution views of the membrane surfaces of cell membranes. We combined fracture-flip with post-fracture extraction of unfractured membranes by Triton X-100. This new version of fracture-flip allows exposure and imaging of the inner (cytoplasmic) surfaces of plasma membranes and, also, of the exoplasmic surfaces in double-membrane organelles. We combined this method with immunogold cytochemistry to study the ultrastructure of the inner surface of the red cell membrane as well as the planar distribution of Band 3 and spectrin (K. Fujimoto). Fracture-flip Triton X was also used to investigate the ultrastructure of nuclear pores in isolated rat liver nuclei (K. Fujimoto). In other studies, we were able to show that the presence of a surface meshwork of 44 nm fusiform filaments characterizes the infective stage of *Leishmania major*. We proposed that these filaments represent metacyclic developmental lipophosphoglycan (P. Pimenta; D. Sacks; R.P. da Silva). We investigated and were able to differentiate the ultrastructure and lectin cytochemistry of the main developmental stages of *Trypanosoma cruzi* (P. Pimenta, W. de Sousa, T. Souto-Padron). We continued the investigation of the surface ultrastructure of developing nerve cells in culture (C. Andersson-Forsman). Studies now in progress address: 1) the surface ultrastructure of H9 cells infected by HTLV-II(3) (S. Ru-Long); 2) the surface ultrastructure and distribution of specific proteins in sea urchin spermatozoa at different stages of development (S. Ru-Long, D. Nishioka); 3) surface ultrastructure of *Giardia lamblia* (P. Pimenta, W. de Sousa); 4) surface ultrastructure and molecular cytochemistry of lymphocyte capping of CD3, CD4 and HL-A antigens (M.R. Torrisi, A. Pavan); 5) surface ultrastructure of *Mycobacterium leprae* and *M. avium* (A. Aguas, M. Teixeira da Silva; see Lepra, new project); and 6) surface microanatomy of *Schistosoma mansoni* collected during its life cycle (P. Pimenta, A. Sher).



## PROJECT DESCRIPTION

Major Findings:

Fracture-flip is a new method to reveal the ultrastructure of membrane surfaces at close to macromolecular resolution. For fracture-flip cells are freeze-fractured and carbon (but not platinum) is evaporated onto the fractured face. The specimens are thawed, washed repeatedly in distilled water and the carbon replicas (with the exoplasmic halves of membranes attached) are then "turned upside-down ("flipped"). The actual surfaces of membranes (from the exoplasmic halves of the membrane) are now on top. After air-drying, they can be imaged at high resolution by evaporation of platinum. In its original form, fracture-flip can only reveal the outer surfaces of cells (or the cytoplasmic surfaces of isolated organelles). We have now modified the method to use post-fracture Triton X extraction of specimens. Triton X dissolves unfractured membranes, but does not extract fractured membrane "halves" because these have their apolar regions already stabilized by carbon (evaporated immediately after fracture). We have now used this new method to provide extensive views of the inner surface of erythrocyte membranes, before and after extraction of spectrin. We have also combined this new method with replica staining techniques (see Label-Fracture; Z01 CB 08378-05 LTB) to identify spectrin molecules at the inner surface (K. Fujimoto). We used also fracture-flip and fracture-flip/Triton X to study the cytoplasmic surface of the outer membrane as well as the exoplasmic surface of the inner membrane in isolated rat liver nuclei (K. Fujimoto). We showed that, contrary to previously thought, the nuclear pore is not lined at its cytoplasmic surface by ribosome-like particles but is, rather, a fairly inconspicuous structure almost "flush" with adjacent areas of the nuclear envelope. In contrast, examination of the exoplasmic surface of the inner nuclear envelope membrane (exposed after Triton X extraction) shows the presence of large globular structures which span the intermembrane (perinuclear) space.

We continued the application of fracture-flip to the study of the surface ultrastructure and topochemistry of the agents of parasitic diseases. These organisms are all characterized by the exquisite dynamics of their cell surfaces which they use to evade the defense mechanisms of their hosts. In *Leishmania major*, we found that characteristic fusiform filaments (avg. length: 44 nm) cover the surface of cells fixed at an infection stage (P. Pimenta, R.P. da Silva, D.L. Sachs). Preliminary immunogold labeling experiments as well as correlation with biochemical data on isolated membranes led us to propose that these filaments represent lipophosphoglycan that is present only in infective promastigotes. Fracture-flip was also used to lead to the ultrastructural differentiation of the main developmental stages of *Trypanosoma cruzi* (promastigotes, amastigotes and epimastigotes) (P. Pimenta, W. de Sousa, T. Souto-Padron). The images that we obtained have a resolution much higher than that provided even by high resolution scanning electron microscopy. For instance, in epimastigotes fracture-flip clearly reveals the presence of a sharply delimited, raised, roughly textured platform at the cytostome. This platform remains invisible even in attempts to visualize with still experimental high resolution SEM (K. Tanaka). We combined replica-staining (see Project Z01 CB 08378-05) with fracture-flip to differentiate the three main developmental stages of the life cycle of *T. cruzi*. Of interest is the very sparse labeling of epimastigotes with the exception of the cytostome which is heavily labeled by concanavalin A, but not labeled by *Ricinus communis* or by *Wistaria floridi-*



bunda lectins. Recently, we started to explore the application of fracture-flip combined or not with immunogold labeling to observe the budding of HTLV-II(B) from H9 cells (S. Ru-Long). We have found that only a small proportion of the cells (less than 3%) show viral particles budding from their surface. However, those that do, do it actively. We note that our objective is not to establish elements on the ultrastructure of the virus, rather to see its surface distribution, mode of budding, mechanism of fusion and detection and mode of distribution of viral antigens (we will start with GP120 and GP41) on cell surfaces. This work will involve conventional freeze-fracture, label-fracture and immunogold labeling of fracture-flipped specimens). WGA labeling of fracture-flipped specimens shows a higher density of receptors on cells producing virus. In addition, we find that WGA does not label the virus particle itself, with labeling more concentrated within an annular area of the plasma membrane surrounding the emerging virus.

#### Publications:

Andersson-Forsman C, and Pinto da Silva P. Fracture-flip: new high resolution images of cell surfaces after carbon stabilization of freeze-fractured membranes. *J Cell Sci* 1988;90:531-541.

Andersson-Forsman C, and Pinto da Silva P. Fracture-flip views the surface of sperm cells macromolecular resolution. Proceedings of the Ninth European Congress of Electron Microsc. York, England; Inst. Phys. Conf. Ser. 93, Chapter 17, 1988;3:467-468.

Andersson-Forsman C, and Pinto da Silva P. The surface of human blood cells as revealed by fracture-flip. Proceedings of the Ninth European Congress of Electron Microsc. York, England; Inst. Phys. Conf. Ser. 93, Chapter 17, 1988;3:477-478.

Andersson-Forsman C, and Pinto da Silva P. High resolution surface views of spermatozoa as revealed by fracture-flip. *J Cell Sci* 1989;92:415-425.

Fujimoto K, and Pinto da Silva P. Macromolecular dynamics of the cell surface during the formation of coated pits is revealed by fracture-flip. *J Cell Sci* 1988;91:161-173.

Fujimoto K, and Pinto da Silva P. The surfaces of nuclear envelope membranes as revealed by fracture-flip. *Eur J Cell Biol*, In press.

Fujimoto K, and Pinto da Silva P. Fracture-flip/Triton X-100: a new method to study the ultrastructure and topochemistry of the cytoplasmic surfaces of biomembranes, Submitted.

Pimenta PF, da Silva RP, Sacks DL, and Pinto da Silva P. Cell surface nanoanatomy of *Leishmania major* as revealed by fracture-flip. A surface network of 44 nm fusiform filaments identifies infective developmental stage promastigotes. *Eur J Cell Biol*, In press.

Pimenta PF, de Sousa W, Souto-Padron T, and Pinto da Silva P. The cell surface of *Trypanosoma cruzi*: a fracture-flip, replica-staining label-fracture study, Submitted.

Pinto da Silva P, Andersson-Forsman C, and Fujimoto K. Fracture-flip: where freeze-fracture is turned upside down and comes from behind the looking glass to reveal the cell surface. Proceedings of the Ninth European Congress of Electron Microsc. York, England; Inst. Phys. Conf. Ser. 93, Chapter 16, 1988;3:451-452.

Pinto da Silva P, Andersson-Forsman C, and Fujimoto K. Nanoanatomy and topochemistry of cell surfaces. In: Motta P ed. In Celebration of Camillo Golgi, In press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03388-02 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Expression of Beta (1-4) Galactosyltransferase

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

Pradman K. Qasba, Ph.D. Research Chemist LTB, NCI

Other Professional Personnel:

Arni Masibay, Ph.D. IRTA Fellow LTB, NCI

Elizabeth Boeggeman, Ph.D. IRTA Fellow LTB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## 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.)

Beta (1-4) galactosyltransferase enzyme (GT) is localized in the trans Golgi where glycoproteins get galactosylated. This enzyme has also been found at the cell surface where it acts as cell-adhesion molecule, mediating intercellular adhesion by binding to terminal N-acetylglucosamine residues on lactosaminoglycans substrates, there by bridging cells together, and as a high affinity receptor for the laminin long arm. The cell surface and Golgi transferase seem to be differentially regulated, the former is induced by beta-adrenergic agonist like isoproterenol without effecting the Golgi transferase while as upon differentiation of certain cell types it is the Golgi transferase that increases without effecting the cell surface transferase. The cell surface GT is possibly involved in growth since its effector molecule alpha-lactalbumin blocks the growth of cells induced by beta-adrenergic agonists isoproterenol. In the current studies we are investigating the expression of GT in several cell lines and the distribution of this enzyme in Golgi and cell surface, hormones involved in the regulation of expression of the two forms of the enzyme and the time when they are expressed during cell-cycle. Our results indicate that in Swiss mouse 3T3 cells GT is expressed prior to DNA replication and then down-regulated.

Project Description:

Expression of beta (1-4) galactosyltransferase (GT) is known to be hormonally regulated. In mammary gland its expression is modulated by lactogenic hormones and is also dependent on the functional differentiation of the mammary tissue. In parotid glands transferase activity is also known to be modulated by beta-adrenergic receptor agonist. In the present study hormonal requirement and tissue specific expression of cell surface and Golgi forms of GT are being investigated.

Major Findings:Expression of Alpha-Lactalbumin, the Effector Molecular of Beta (1-4) galactosyltransferase in the Mammary Gland:

Epidermal growth factor (EGF) markedly enhances the selective, prolactin-independent induction of alpha-lactalbumin in mammary explants from pregnant rats. Commercial preparations of serum albumin from six species contain a small molecule with similar effects and this molecule is sensitive to increased temperature and resistant to trypsin digestion. Of the five milk protein mRNAs studied, only alpha-lactalbumin is induced by insulin, glucocorticoid and serum albumin in the absence of prolactin. The biological effects of serum albumin contaminant and EGF through similar, they appear to operate by different mechanisms since their effects on the rat system are additive.

Cell-cycle dependent expression of Beta 1-4 galactosyltransferase in mouse 3T3 cells:

Expression of 1-4 galtransferase during different stages of cell-cycle in mouse 3T3 cells is studied in conjunction with the expression of other mRNAs during the cell's transit through G<sub>1</sub>. 1-4 galtransferase is expressed during early G<sub>1</sub> period of these cells (2.5 hrs after serum stimulation of the confluent cells)<sup>1</sup>. These mRNA levels then decline by the 6th to 8th hour of stimulation. Other mRNA species stimulated during this period have been identified. One of them being gamma actin-specific mRNA.

Expression of Beta 1-4 galactosyltransferase in established cell-lines:

Expression of galtransferase is being studied in various tissues and cells lines. In the present study we are screening several cells lines for 1) the activity of 1-4 galtransferase, 2) the molecular form of the protein produced, 3) mRNA levels for the enzyme and 4) mRNA size class produced.

Publications:

Ziska SE, Bhattacharjee M, Herber RL, Qasba PK, Vonderhaar BK. Thyroid hormone regulation of alpha-Lactalbumin: Differential glycosylation and messenger ribonucleic acid synthesis in mouse mammary glands. Endocrinology 1988;123:2242-2248.



Nichols KR, Sankaran L, Kulski JK, Chomczynski P, Qasba PK. Comparison of some biological effects of epidermal growth factor and commercial serum albumin on the induction of alpha-lactalbumin in rat and rabbit mammary explants. J Endocr 1988;119:133-139.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 08389-02 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Structure-Function Relationship of Beta (1-4) Galactosyltransferase

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

Pradman K. Qasba, Ph.D.

Research Chemist

LTB, NCI

## Other Professional Personnel:

Arni Masibay, Ph.D.

IRTA Fellow

LTB, NCI

Elizabeth Boeggeman, Ph.D.

IRTA Fellow

LTB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

1.5

## PROFESSIONAL:

1.5

## OTHER:

0

## CHECK APPROPRIATE BOXES)

 (a) Human subjects (b) Human tissues (c) Neither (a1) Minors

B

 (a2) Interviews

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

Our ongoing studies on the structure-function relationship of proteins, specifically of glycosyltransferases and their modifiers like alpha-lactalbumin, continued this year. We report for the first time that the cDNA sequence of bovine beta (1-4) galactosyltransferase (GT) (constructed from a partial cDNA clone and a genomic fragment) engineered in an Okayama-Berg vector, upon transfection of COS-7 cells, codes for an enzymatically active GT.

There is an approximately 12 fold increase in the GT activity upon transfection of COS-7 cells with sense cDNA compared to antisense or pSV,Neo or mock transfected cells. Polyclonal and monoclonal antibodies directed against synthetic peptide corresponding to the amino-terminal region of the GT protein, bind the expressed protein and the immunoprecipitates exhibit GT enzymatic activity. The expressed GT activity is modulated by alpha-lactalbumin to change the acceptor specificity to glucose, to synthesize lactose. These results show that the expressed GT protein is fully functional and that the binding sites for UDP-galactose, N-acetylglucosamine, glucose and alpha-lactalbumin are all intact and operative. We have also engineered the cDNA sequences of galactosyltransferase and alpha-lactalbumin, in such a way that the expression of the cDNA sequences have a potential to produce either soluble and secreted forms or membrane anchored forms of the protein.

Project Description:Objective:

To Determine a "Functional" Region or a Unit of a Protein.

Using the methodology of the recombinant DNA technology, we are developing general methods to produce the normal and altered proteins. Mammalian and prokaryotic expression vectors, and Baculovirus vector systems are engineered in such a way that the same vector can be used to produce protein in quantities enough to test its function, and as well to produce the engineered protein in large quantities for determining various physical properties and X-ray crystallographic studies.

Major Findings:

Assembly of the DNA Sequence of Alpha (1-4) Galactosyltransferase (GT) and Construction of GT Expression Vector:

A full-length bovine GT cDNA was constructed from a partial cDNA clone and a genomic fragment containing 5'-end sequences of the cDNA. The bovine genomic library was screened with a synthetic 100-mer oligonucleotide corresponding to the extreme 5'-end sequence of a longest GT cDNA clone which lacked the translational start codon. The DNA restriction fragment carrying the 5'-end of the cDNA was isolated, and sequenced to ensure that it contained sequences covering the 5'-end region of the GT gene. The sequence data showed that the DNA encodes the 18 amino-terminal end residues of the bovine GT which include the two in-frame methionines. The full-length cDNA was assembled on the Okayama-Berg vector where its expression is under the control of SV40 promoter. The resulting vectors, pLsGT and pLasGT contain GT cDNA in the sense and antisense orientation respectively.

Expression of Bovine GT in COS-7 Cells:

Cos-7 cells were transfected with pLsGT, pLasGT, pSV<sub>2</sub>neo DNA or mock transfected. The synthesis of the RNA from these vector in the transfected cells was monitored with the 3'-end DNA probes. The GT activity was measured from the cell extracts.

Activity of the Expressed Bovine GT: Modulation of the Activity by Alpha-Lactalbumin:

There is about 12 fold increase in the activity of the extracts assayed in the presence of N-acetylglucosamine (NAG) as an acceptor substrate, from pLsGT transfected cells compared to the extracts from pLasGT- a vector carrying GT cDNA in antisense orientation -, pSV<sub>2</sub>neo DNA or mock transfected cells. This increase in the activity corresponds to a 60 fold stimulation when assayed in the presence of NAG as an acceptor over the samples assayed in its absence, showing that the activity is absolutely dependent on the added exogenous acceptor substrate. The expressed GT protein in pLsGT transfected COS-7 cells

can be modulated by bovine alpha-Lactalbumin to accept glucose as a substrate to produce lactose. There is a 10 fold increase in the lactose synthetase activity in the extracts from pLsGT transfected cells compared to the extracts from controls. These results show that the bovine GT protein expressed transiently in COS-7 cells and coded by the cDNA is fully functional and that the binding sites for UDP-galactose, NAG, glucose and alpha-lactalbumin are all intact and operational.

#### Immunological Identification of Bovine GT Expressed from cDNA:

The enzyme in the transfected cells from the sense GT cDNA is precipitated by the polyclonal antibody raised in rabbits against SDS-PAGE purified bovine milk GT. The immunopellets obtained by the precipitation of the antigen-antibody complex with the second antibody, showed GT enzymatic activity.

A mouse monoclonal antibody directed against a fifteen residue long synthetic peptide, corresponding to the amino-terminal region of the cDNA encoded GT protein, binds the expressed bovine GT and the immunoprecipitates exhibit GT enzymatic activity.

#### Engineering of the cDNA Sequences of 1-4 Galactosyltransferase and alpha-lactalbumin:

We have engineered the GT cDNA sequence in such a way that some of the sequences have a potential to produce a 320-residue long soluble and secretory form of the enzyme, and others a soluble form of enzyme with alpha-lactalbumin signal peptide sequence and others a 401-residue long membrane anchored form of the protein. These constructs are being used for the localization of these and other altered GT's in the cell and for the assignment of various functional and interacting domains of GT protein.

#### Publications:

Masibay AS, Qasba PK. Expression of bovine beta (1-4) galactosyltransferase cDNA in COS-7 cells. Proc Natl Acad Sci, 1989, vol. 86.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08390-01 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (50 characters or less. Title must fit on one line between the borders.)

Analysis of HIV Assay Kinetics

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

John L. Spouge, M.D. Ph.D.

Visiting Scientist

LTB, NCI

Other Professional Personnel

Peter Nara, VCS, NCI, Frederick Cancer Research Facility

## COOPERATING UNITS (if any)

Scott Layne, T-7, Theoretical Division, Los Alamos National Laboratory;

Micah Dembo, T-10, Theoretical Division, Los Alamos National Laboratory.

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

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.)

HIV assays have not yet been standardized, and a comparison between assays, although important, is not yet dependable. For example, a series of five papers in Nature evaluating soluble CD4 (sCD4) as a blocker of HIV infectivity were divided two and three, for and against the efficacy of the blocking properties of sCD4. With these results in mind, we developed a theory to account for the hidden variables influencing the outcome of the experiments.

In our model, four unknown parameters characterize the virus under assay conditions: shedding and inactivation rates, an infection rate, and the association constant for gp120-sCD4 interaction. These parameters can be determined by experiments following certain protocols. Peter Nara is currently carrying out some of these protocols at FCRF, and the results show that viral infectivity assays and physical methods produce similar estimates of the gp120-sCD4 association constant. This is a considerable saving of experimental effort since the viral infectivity assays are much less laborious than the physical methods. Further experiments characterizing various viral strains and their tropism for different cell lines are planned.

Project Description:Major Findings:

Much work to verify the assumptions of the model remains, but preliminary findings support the assumption that viral infection proceeds at a rate directly proportional to the number of gpl20's remaining on a virion, i.e, a virion with 80 gpl20's is twice as infectious as a virion with 40 gpl20's. This assumption is crucial in determining the association constant of gpl20-sCD4 by infectivity assay. Determinations based on the infectivity assays published in Nature when analyzed with our model, are compatible with values from physical methods.

Publications:

Layne SP, Spouge JL, Dembo M. Quantifying the infectivity of HIV. PNAS, in press.

Spouge JL, Dembo M, Layne SP. Analytic results for a model describing blocking of HIV infection. Bull Math Biol, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08391-01 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Improving Sequence Alignment Algorithms

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

John L. Spouge, M.D., Ph.D.

Visiting Scientist

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.

0.5

## PROFESSIONAL:

0.5

## OTHER:

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.)

Because of the increased size of databases, computer processing of sequence data requires efficient algorithms. Probably the most frequently used algorithms are variants of the Needleman-Wunsch algorithm, used to align and compare two sequences. Comparison of more than two sequences is a problem of current interest, but this is even more computationally expensive.

A theory relating alignment efficiencies and inequalities directly was used to develop several "new" algorithms, which were then tested empirically on the Laboratory mini-computer. One of these was shown to have a clear superiority in both time and memory requirements and is the fastest known algorithm for aligning two sequences under Needleman-Wunsch single indel weight penalties. Similar new algorithms allowing multiple indel penalties (which are biologically more realistic) were also tested and one was shown to be the best alignment algorithm under multiple indel weights currently available. The theory is also applicable to multiple sequence alignment.

Project Description:Major Findings:

When scrutinized critically, previous improvements on the Needleman-Wunsch algorithm, notably those of Fickett and Ukkonen, were based on inequalities. Pursuing this observation methodically led to a theory which relates alignment efficiencies and inequalities directly. This theory was used to develop several "new" sequence alignment algorithms, which were then tested empirically. One of these was shown to have a clear superiority in both time and space requirements over the Fickett and Ukkonen algorithms and is therefore the fastest known algorithm for aligning two sequences under the Needleman-Wunsch single indel penalty scheme. Similar new algorithms allowing multiple indel penalties (which are biologically more realistic) were also tested. One of these was superior to the Miller-Myers algorithm, currently the best alignment algorithm under multiple indel weights. Both improved algorithms have been written up and are being refereed.

Moreover, the theory has ramifications far beyond sequence alignment. Almost any physical law (Newton's laws, Fermat's principle in optics, Hamilton's principle in mechanics) can be rephrased as a problem from the calculus of variations. When discretized, problems from the calculus of variations become problems in lattice path optimization. The theory can be used to speed up any dynamic programming optimization of a lattice path, and so may have considerable other uses. In biological applications, multiple sequence alignment can be so reduced to optimization of a lattice path and the theory therefore provides a convenient language, that of inequalities, for discussing improvements in multiple sequence alignment algorithms.

Publications:

Spouge JL. Speeding up dynamic programming algorithms for finding optimal lattice paths. SIAM J Appl Math, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08392-01 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the brackets.)  
Combination Chemotherapy of AIDS and Cancer

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

Janos Szebeni, M.D.

Visiting Associate

LTB, NCI

Gurupaddapa Betageri

Visiting Fellow

LTB, NCI

Vellarkad N. Viswanadhan

Biotechnology Fellow

LTB, NCI

Kenneth Hung

Summer Student

LTB, NCI

## COOPERATING UNITS (if any)

LTCB, DCBD, NCI; LMI, NIDR; O.D., DCE, NCI; Dept of Pathology, Uniformed Services Univ. of Health Sci.; P.R.I., NCI/FCRF

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Theoretical Immunology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS.

3.3

## PROFESSIONAL.

3.0

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

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

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

A new approach to combination chemotherapy of AIDS has been developed in the Theoretical Immunology Section. Dipyridamole (DPM) is widely used as an oral agent for cardiovascular indications because of its platelet antiaggregant and coronary vasodilation activities. We recently found that DPM potentiates the activity of azidothymidine (AZT) and other dideoxynucleosides against human immunodeficiency virus (HIV-1) in cultured human monocyte/macrophages and stimulated T-lymphocytes. In cultured human lymphoblastoid cells, DPM potentiates the antiviral activity, and it also protects the cells from AZT's cytotoxic effects. DPM does not potentiate the cytotoxic effect of AZT on human bone marrow progenitor cells in vitro. These findings suggest the possibility that DPM might increase the therapeutic index of AZT and, perhaps, other dideoxynucleoside agents in vivo. However, the in vitro results cannot be used to predict clinical efficacy or safety. AZT/DPM has been approved for study by the AIDS Clinical Trial Group. Phase I trials are being planned, in conjunction with collaborators. Other aspects under study:

1. Mechanism: DPM blocks cellular uptake of physiological nucleosides but not of AZT. The potentiation of AZT may thus result, in part, from decreased influx of the nucleosides that compete with AZT for viral reverse transcriptase.
2. Molecular structure: A structure for DPM has been computed from the crystallography. Quantitative structure-activity relationships are being studied to predict which features of this class of molecules are required for activity.
3. Analysis of combination chemotherapy: Because no published algorithm or computer package was adequate for analysis of our data on the antiviral effect of drug combinations, we have developed a new approach. The computer program package, called COMBO, will be useful in the context of cancer as well as AIDS.

Project Description:Major Findings:

1. Dipyridamole (DPM) modestly inhibits replication of HIV-1 in cultured human monocyte/macrophages; more strikingly, it potentiates the anti-HIV activity of AZT and other dideoxynucleosides in those cells.
2. DPM potentiates AZT against HIV-1 in human T-lymphocytes (i.e., phytohemagglutinin-stimulated, IL2-stimulated mononuclear cells).
3. DPM potentiates AZT against HIV-1 in a T-lymphoblastoid cell line (CEM-SS) and also protects those cells against the cytotoxic effects of AZT. Thus, the "in vitro therapeutic index" for those cells is greatly increased.
4. DPM does not potentiate the toxicity of AZT for human bone marrow progenitor cells in a CFU<sub>GM</sub> assay.
5. DPM inhibits uptake of thymidine by monocyte/macrophages, whereas it does not inhibit uptake of AZT. This "differential transport inhibition" may be one of the mechanisms underlying the potentiation of AZT activity, but other effects are probably operating as well.
6. DPM has been reported to induce interferon, but we find no such induction in our studies. We do find, however, that HIV-infected monocyte/macrophages produce alpha-interferon, at least part of which is acid-labile. This interferon may give rise to some of the debilitating symptoms of AIDS, and it has been reported as an early predictor of the onset of clinical disease in infected individuals.
7. DPM does not bind as strongly to serum proteins as has been suggested by less extensive studies in other laboratories. This finding is favorable with respect to the possibility of achieving clinically effective concentrations in vivo.
8. A new algorithm has been developed (in collaboration with Dr. B. Bunow, Civilized Software, Inc.) for analysis of data on drug synergy and antagonism. The method starts with a series of expressions based on enzyme kinetic models and heuristic principles. A program package called "COMBO" was developed to perform the following tasks, operating in the MLAB computing environment: (i) globally fit each model by least squares regression with constant weights; (ii) use the results in a windowing technique to generate updated weights; (iii) repeat regression with the updated weights; (iv) iterate the previous two steps; (v) use a combination of normal theory and Monte Carlo techniques to determine confidence limits on the fitting parameters; (vi) construct a set of derived parameters that express various aspects of synergy, potentiation, and antagonism; (vii) calculate confidence limits on those constructed parameters; (viii) produce graphical displays of the error model, the residuals of each fit, and the contours of equipotent drug effect.

Publications:

Szebeni J, Wahl SM, Popovic M, Wahl LM, Gartner S, Fine RL, Skaleric U, Friedman RM, Weinstein JN. Dipyridamole potentiates the inhibition by azidothymidine and other dideoxynucleosides of human immunodeficiency virus replication in monocyte/macrophages. Proc Natl Acad Sci USA 1989;86:3842-3846.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08393-01 LTB

PERIOD COVERED  
October 1, 1988 to September 30, 1989

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

The Leprosy Bacillus: Structure and Cell Biology

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

P. Pinto da Silva, Ph.D. Chief LTB NCI

Others: S. Ru-Long, M.D. Visiting Fellow LTB NCI

D. Taylor, M.S. Research Associate LTB NCI

(2/20/89 to 6/30/89)

COOPERATING UNITS (if any)

Institute of Experimental Cytology, University of Porto, Portugal (A. Aguas, M. Teixeira da Silva)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

National Cancer Institute, Frederick Cancer Research Facility

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews

B

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

We are studying the molecular organization of the cell envelope of the leprosy bacillus (*Mycobacterium leprae*) by the analysis of high-resolution freeze-fracture images of the bacteria. The crystalline patterns found in these micrographs will be characterized by laser optical diffraction. Because *M. leprae* is noncultivable *in vitro*, liver fragments from infected armadillos and nude mice are used. We are comparing the structure of the envelope of the leprosy bacillus with that of other pathogenic mycobacteria: *M. lepraemurium* and *M. avium*. It is our aim to investigate the structural basis of mycobacterial escape from destruction inside the macrophage.



## PROJECT DESCRIPTION

Major Findings:

The major goal of our project is to study the structure of *Mycobacterium leprae* and the interaction of the mycobacteria *in vivo* with the macrophage in experimental animals and in human patients. This mycobacterium is responsible for leprosy, a disease that severely impairs 12 to 15 million human beings with no effective vaccine available, and with a therapy that often is of only incomplete success. Infections by other mycobacteria are also under study, in particular those induced by *M. lepraemurium*, the agent of murine leprosy and *M. avium* that is responsive for frequent and often fatal infections in AIDS patients. The experimental mycobacterial infections are conducted by Dr. A. Aguas and by Professor Teixeira da Silva at the Center for Experimental Cytology, Porto, Portugal. The tissue specimens sent from Portugal are used to study by freeze-fracture electron microscopy the molecular organization of the different layers that make up the cell envelope of the leprosy bacillus. The results will be compared with those obtained from freeze-fracture experiments of other mycobacterial species and will integrate data obtained by "fracture-label" and thin section electron microscopy to be obtained by our Portuguese colleagues.

Our preliminary freeze-fracture experiments reveal that the cell envelope of mycobacteria comprises multiple layers of lipids in crystalline arrays with a hexagonal patterning. The discovery of this repetitive arrangements prompted us to start a collaborative effort with the Electronics Department of the Engineering Faculty of Porto in order to define the precise molecular arrangement of these crystalline arrays by laser optical diffraction. We have also found out that the different mycobacterial species (*M. leprae*, *M. lepraemurium*, *M. avium* and *M. smegmatis*) studied during *in vivo* infections in mice show distinct intracellular fates in liver and peritoneal macrophages. Most of *M. leprae* bacilli were seen in the cytoplasmic space without any membrane lining. *M. lepraemurium* were seen inside phagosomal vesicles, but produced a compact layer of cylinders or tubules that separated them from the remainder contents of the phagosome. *M. avium* may also synthesize a capsule-like material inside the phagosome, and *M. smegmatis* was invariably seen unprotected inside the macrophage phagosomes.

At a later stage of the project, we plan to study the biochemical composition and the structural arrangement of the phagosomal membrane that limits intracellular mycobacteria. This is because one of the mechanisms that has been postulated to explain the survival of mycobacterium inside the hostile environment of the macrophage is a mycobacteria-induced inhibition of lysosome-phagosome fusion in infected macrophages. Our hypothesis is that the mycobacteria will in some way change the structure and/or composition of the phagosomal membrane so that its cytoplasmic side becomes refractory to lysosomal fusion.

Publications:

New project; no publications.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08394-01 LTB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Non-Contiguous Patterns and Functional Domains in DNA.

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

Andreas K. Konopka, Ph.D.,	Visiting Scientist	LTB, NCI
Devjani Chatterjee, Ph.D.,	Visiting Associate	LTB, NCI
John Owens,	Computer Specialist	LTB, NCI

## COOPERATING UNITS (if any)

Harvard School of Public Health, MBCRR (Dr.G.Mengeritzky)  
 Weizmann Institute of Science, Dept. Polymer Research (Dr.E.N.Trifonov)  
 Stanford University, Dept. Mathematics (Dr.S.Karlin)

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.95

## PROFESSIONAL:

1.3

## OTHER:

.65

## CHECK APPROPRIATE BOX(ES)

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

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

The vast majority of sequence analysis programs available to date are designed to allow users to detect contiguous sequence fragments that occur more frequently than other contiguous motifs. However, substantial experimental evidence indicates that specific functions of nucleic acids fragments are rarely associated with single, simple sequence motifs. The patterns responsible for biological function are most often non-contiguous and consist of several short oligonucleotides separated by gaps of variable length and composition. In view of the ongoing large sequencing projects reliable procedures for physical mapping of DNA functional domains will be invaluable. To make reliable maps one needs reliable criteria that distinguish between sequences involved in different functions. Non-contiguous pattern analysis already has provided a substantial number of criteria for distinction between protein coding genes, introns, and illegitimate recombination sites. It is believed that by using this methodology various kinds of regulatory regions within repetitive DNA sequences will be mapped unambiguously.

Project Description:Major Findings:

At present extensive statistical analyses of large collections of sequences that have variable length and composition are performed on the CRAY. The FORTRAN programs written for this purpose (in the period 1986-1989) can be divided into three categories:

- 1) Detecting statistically significant occurrence of contiguous patterns.
- 2) Analysis of occurrence of non-contiguous patterns and
- 3) Predicting putative biological roles of any user-specified sequence.

The programs utilize libraries of functionally equivalent sequences (Introns, Exons, Bacterial genes, tRNAs, Spacers, Upstream regions, Downstream regions, Recombination sites and so on). For this reason we developed a procedure to keep our libraries of sequences in a form suitable for statistical analyses.

The following are the most important results obtained thus far from the analysis of large collections of functionally equivalent sequences:

- 1) Intron sequences are generally non-random which is in contradiction with the former prevailing view.
- 2) Several non-contiguous patterns (like two base periodicity or clustering of dinucleotides) are 'intron specific' and do not occur in exons or non-alu repeated DNA sequences.
- 3) Exons and bacterial genes also display periodic non-contiguous patterns but the period is three bases (and not two as in introns).
- 4) Introns might be the hot-spots for illegitimate recombination since several similar non-contiguous patterns have been observed in the sequences of eukaryotic non-homologous recombination sites.
- 5) Compositional complexity of intron sequences is generally lower than in exons. This effect is strong enough to permit correct mapping of introns in pre-mRNA sequences.

Publications:

Konopka AK, Chatterjee D. Distance analysis and sequence properties of functional domains in nucleic acids and proteins. Gene Anal Techn 1988;5:87-93.

Konopka AK, Owens J. Non-contiguous patterns and compositional complexity of nucleic acid sequences. In: Bell G, Marr T. (Eds) Computers and DNA. Addison-Wesley Longman, in press.

Konopka AK, Owens, J. Charts can be used to map functional domains in DNA. Gene Anal Techn 6, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08395-01 LTB

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Somatic Cell Non-homologous Recombination

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

Andreas K. Konopka, Ph.D.,	Visiting Scientist	LTB, NCI
Jacob V. Maizel, Ph.D.,	Chief, Lab. Math. Biol.	LTB, NCI
Akinori Sarai, Ph.D.,	Visiting Scientists	LTB, NCI

COOPERATING UNITS (# any)

Lab. Molecular Biol. MPI, Goettingen, West Germany (Dr. Thomas Jovin)  
Univ. of Wisconsin, Dept. Physiological Chemistry (Dr. Han Htun)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.05

PROFESSIONAL:

1.05

OTHER:

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.)

Rare DNA rearrangements that require no extended sequence similarity (of parental molecules) can cause severe genetic alterations of chromosomal fragments in somatic cells. Thus far about 600 parental sequences for this kind of crossing-over are known. It is expected that their number will increase with the rate 50-100 per year. The purpose of this new project is to store available data on illegitimate recombination in one place. Another goal is to provide simple tools for experimental molecular biologists to decide if newly sequenced DNA fragments contain potential recombination 'hot-spots' or not. The research part of this project concerns analysis of known sequences and possible elucidation of mechanisms for non-homologous recombination. Methods of sequence analysis and molecular modeling have been applied thus far.

Project Description:Major Findings:

Studies of sites stored in our database have shown that Topoisomerase I cleavage sites and runs of purines are major sequence features in the close vicinity of the breakpoint. It has also been shown that those parental sequences that include five or more contiguous purines are associated with at least one Topo I cleavage site. This and other evidences suggest that unusual (non-B) DNA structures might serve as intermediate in many instances of non-homologous recombination. Thus far we have studied one such unusual DNA structure: DNA tetraplex with purine strands in parallel orientation. We were able to show that this structure and the Holiday junction can be resolved in a similar way.

Publications:

Konopka AK. Compilation of DNA strand exchange sites for non-homologous recombination in somatic cells. Nucleic Acid Research 1988;16:1739-1758.

Konopka AK. Sarai A, Htun H, Maizel JV. CSH meeting on intermediates in non-homologous recombination. 1988;169.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08396-01 LBT

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Information Content of Molecular Binding Sites

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

Thomas D. Schneider

Staff Fellow

LTB, NCI

## COOPERATING UNITS (if any)

John Spouge, NLM (Bethesda); Peter Basser, DRS, BEIB (Bethesda); Joe Mack, BRI, Crystallography Laboratory (Frederick); Peter Rogan, BRI, Laboratory of Eukaryotic Gene Expression (Frederick).

## LAB/BRANCH

Laboratory of Mathematical Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, FCRF, Frederick, MD 21701

## TOTAL MAN-YEARS.

1.0

## PROFESSIONAL:

1.0

## OTHER:

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.)

This project is an attempt to find a fundamental theory of how macromolecules operate. The study began by asking how molecules such as repressors, polymerases and ribosomes are able to locate specific patterns (binding sites) on DNA or RNA. The methods of information theory so successfully used by electrical engineers and computer scientists were applied to this problem. Using these tools, we can show quantitatively that the amount of pattern in the binding sites is just sufficient for the sites to be located in the genome (Schneider 1986, Schneider 1988, Schneider 1989). To explain these results, a general theory is being developed that quantitatively explains the precision macromolecules exhibit while performing their tasks. The theory ties Shannon's communications theory to molecular biology.

The techniques being formulated in this project include pattern searching techniques that are more precise than the well known "consensus sequence", and a general cloning technique that can be used to quantitatively define the patterns of a binding site or other functional region of nucleic-acid sequence.

The project involves three lines of work: (1) analysis of known DNA or RNA binding site patterns to test the hypothesis that the pattern at binding sites is related to their number in the genome; (2) bench work to dissect those binding sites which provide critical tests of the theory; and (3) extension of the theory to describe all specific molecular interactions.

Project Description:Major Findings:

Genetic regulation always requires patterns of nucleotide sequences in DNA or RNA. The complexity of these patterns can be described in terms of the number of bits of information 'stored' in them. (A bit is the amount of information needed to distinguish between two equally likely possibilities.) For example, ribosome binding sites carry an average of 11.0 bits of information. The ribosome must be able to distinguish its binding sites from all of the other RNA patterns in the genome. From the size of the genome and the number of sites, we can predict that ribosomes need at least 10.6 bits of information to find the sites. Thus there are two independent measures that are strikingly close. This is true not only for ribosome binding sites, but also for other genetic control systems. Thus the binding site pattern complexity (information) appears to be just sufficient for the pattern to be found in the genome. I hypothesized that binding sites from all organisms (procaryotic, eucaryotic and viral) should always contain the amount of information that is needed for the sites to be located, but not more.

There is an extraordinary exception to this hypothesis. When bacteriophage T7 infects *E. coli*, it replaces the host polymerase with a new polymerase that binds to a set of promoters on the phage genome. The patterns at these promoters have 35 bits of information, far in excess of the 17 bits that should be needed to locate them. If the excess information is not used by the polymerase, then the polymerase should accept patterns containing less information. To test this, many T7 promoters variants were chemically synthesized and sequenced. The amount of information that the polymerase alone requires is  $18 \pm 2$  bits, which dramatically confirms the original hypothesis. The extra information is likely to be the binding site of another protein.

In the last year, the mathematical theory was extended to establish direct relationships between the foundations of information theory and molecular biology. The extended theory will allow electrical engineers, physicists, thermodynamicists and mathematicians to apply their specialized knowledge directly to many problems in molecular biology.

The theory is about 'molecular machines', which are those macromolecules capable of making distinct choices. For example, EcoRI acts as a molecular machine in three ways: (1) by binding to DNA, (2) by locating the sequence 5' GAATTC 3' on random DNA sequences, and (3) by cutting the DNA. The second 'operation' is, of course, a binding site recognition, so the new theory is an extension of the previous work on binding sites. However, the new theory also describes machine operations that appear at first glance to be very different, such as the capture of a photon by the light sensitive pigment rhodopsin. An excited rhodopsin molecule must be able to avoid going backwards to its original state, rather than forwards to the bathorhodopsin state. (A chemical cascade amplifies the bathorhodopsin state 400,000 times and leads to a nerve impulse. Because of this we can see single photons of light.) Thus rhodopsin must make a tiny



'decision' about what to do, and how it does this may be studied using information theory.

The first major step in constructing the new theory is establishing an upper bound on what a molecular machine is capable of doing, namely its capacity. The formula for machine capacity is related to Shannon's famous channel capacity for communications systems,  $C = W \log_2 (P+N/N)$ . To be sure that I am placing the theory on a rigorous theoretical basis, I have been in close consultation with several NIH scientists both in Frederick and in Bethesda.

Once the machine capacity formula has been determined rigorously, several results immediately follow from Shannon's 'precision theorem'. When applied to molecular machines, this theorem states that so long as the machine capacity is not exceeded, the error rate may be made arbitrarily small. This result is applicable to every macromolecule studied by molecular biologists, and it resolves many apparent mysteries in the current literature. For example, Rosenberg's group has been unable to understand how the restriction enzyme EcoRI is capable of binding precisely to the pattern 5' GAATTC 3', without detectably binding to other hexamers. The reason for this 'specificity' is that EcoRI does not exceed its capacity, so its error rate may be tiny, in accordance with the theorem.

Using this theorem, one can also prove that there is a precise minimum amount of energy that a molecular machine must use to gain one bit of information,  $kT \ln$ . This result is counter to the current thinking by many physicists. In determining this bound, I found a surprisingly deep relationship: the channel capacity is an extension of the second law of thermodynamics. This has been unrecognized for the past 40 years.

The idea of building molecular computers has intrigued many people in the last few years. This theory contributes to that field by showing that single macromolecules can perform precise Boolean logic if they do not exceed their capacity.

#### Publications:

Schneider TD. Information and entropy of patterns in genetic switches. In: Erickson GJ, Smith CR, eds. Maximum-entropy and bayesian methods in science and engineering. Dordrecht, The Netherlands: Kluwer Academic Publishers, 1988; 147-154.

Schneider TD, Stormo GD. Excess information at bacteriophage T7 genomic promoters detected by a random cloning technique. Nucleic Acids Research, 1989;17:659-674.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08906-02 OD

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Studies with Wild-type and Mutant HIV-1 Protease Expressed in E. coli

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

P.I.	John M. Louis	Staff Fellow	OD DCBD NCI
Others:	P.T. Mora	Supervisory Research Chemist	OD DCBD NCI
	C.A.D. Smith	Visiting Associate	OD DCBD NCI
	V. McFarland	Chemist	OD DCBD NCI
	R. McDonald	Student (Intern)	OD DCBD NCI

## COOPERATING UNITS (if any)

S. Oroszlan, Bionetics, NCI, FCRF  
D. Davies, LMB, NIDDK

## LAB/BRANCH

OD, DCBD

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.4

## PROFESSIONAL:

2.15

## OTHER:

0.25

## CHECK APPROPRIATE BOX(ES)

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

B

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

HIV-1, like all other replication competent retroviruses, encodes a protease [PR] which is responsible for the specific cleavage of Pr55<sup>gag</sup> and Pr160<sup>gag-pol</sup> into the mature gag derived structural proteins p17, p24, p7 and p6, and the pol derived protease [PR], reverse transcriptase [RT] and the endonuclease, the integration protein [IN], necessary for the virus maturation. The 297bp gene coding for the HIV-1 PR was chemically synthesized and expressed in E. coli. This method has the advantage for conducting rapid mutational analyses. In order to determine the functional role of the second conserved GlyArgAsn sequence, substitution mutations were introduced into this region (Arg87 -> Lys; Arg 87 -> Glu; Asn 88 -> Glu) of the PR gene using the wild-type clone. The products of the mutant and the wild-type clones were expressed at approximately similar levels at 30 minutes of induction but the mutant PR proteins accumulated as a function of time of induction, unlike the wild-type PR which declined after 60 minutes. The product expressed from the wild-type clones exhibited specific cleavage of gag related polypeptide and peptide substrates spanning different cleavage sites in the gag-pol polypeptide while the mutant clones were completely devoid of proteolytic activity. Further characterization of these and similar mutant PRs may aid in understanding the structure-function relationship of the essential regions and in the design of specific inhibitors capable of recognizing the viral PR but not the related PRs of the host.

Construction of mutant protease clones:

We had earlier shown the expression of the 99 amino acid polypeptide product of the synthetic PR gene in *E. coli*, which corresponds to the 11.5 kDa PR protein in the virions, and the specific proteolytic activity of the expressed product was demonstrated using synthetic substrates. The wild-type clone bearing the correct coding sequence of 297bp was used to introduce mutations in the second conserved region, located at the C-terminal domain of the protease protein. The three mutants that were constructed were Mut3-11 (Arg 87 -> Lys; maintaining a positive charge on the side chain), Mut4-1 (Arg 87 -> Glu; shortened and negatively charged side group), and Mut5-10 (Asn 38 -> Glu; a major change to a negatively charged and longer side chain).

Comparison of the expressed PR of wild-type and mutant clones:

The products expressed in the mutant clones were identical in size and amounts to the wild-type product (11.5 kDa) after 30 min of induction. Immunoblotting of the gene products of the wild-type and mutant clones as a function of time of induction using 1mM IPTG, showed that the expressed product in the wild-type clone accumulated to a maximum at 30 min of induction, after which the level of PR rapidly declined. A similar pattern of accumulation of the product was observed with the reconstructed wild-type clone, with a maximum at 45 to 60 min of induction. In contrast, all the mutant clones showed an accumulation of product as a function of time up to 2h. The amount of PR was two to three fold higher at 2h than after 30 min of induction.

Loss of protease activity by the introduced mutations:

The specific proteolytic activity of the expressed products derived from wild-type and the mutant clones Mut3-11, Mut4-1, and Mut5-10 were analyzed using synthetic peptides, one of them corresponding to the HIV-1 p17-p24 cleavage site in the natural gag polyprotein, as well as using a *E. coli* expressed gag polyprotein substrate. Extracts from induced wild-type clones, showed specific cleavage of the synthetic nonapeptide Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-amide. Based on the kinetics of substrate cleavage, the apparent specific activity was estimated to be 40-50 pmoles substrate per min/ug protein. When using the GAG4 polyprotein, cell lysates derived from induced clone PR-C showed specific cleavages at Tyr/Pro, Leu/Ala and Met/Met to release a major 24 kDa and minor 26 kDa products. Neither the mutant cell extracts which had comparable or higher levels of expressed PR nor the uninduced wild-type extracts were capable of cleaving either the synthetic peptide or GAG4 substrates.

Modeling studies with HIV-1 PR dimer (in collaboration with A. Wlodawer, NCI-FCRF) indicated that the side-chain of the Arginine 87 reaches into the active site and in fact belongs to the cleft floor where the substrate rests and it has been suggested that it might interact by ion pairing with the similarly highly conserved Asp29. Preliminary studies indicate that the substrate was capable of binding to this mutant protease.

Conditions have been established for the purification of the enzyme to more than 90% homogeneity. This has been accomplished by ammonium sulphate fractionation, hydrophobic interaction, pepstatin binding, ion exchange and gel filtration chromatography. Routine screening of compounds for enzyme inhibition studies is now being established (in collaboration with S. Oroszlan and E.M. Wondrak, NCI-FCRF).

Other studies involve understanding the processing of the entire gag-pol polyprotein both in E. coli and mammalian cells to define the order of cleavage of the precursor and the mechanism of self-processing of the protease. Progress is being made in establishing a system for the over expression and rapid purification of the wild-type and the mutant PRs as a fusion to the E. coli maltose-binding protein. The mutant PR co-crystallized with the substrate will be used to study the 3-D structure of the substrate-enzyme complex. The structure function relevance of the region GlyArgAsn especially that of Arg87, which is unique in retroviral proteinases unlike in pepsins, will be further explored. Similar mutational analysis will be carried out to find essential and unique regions of the viral PR which do not occur in mammalian PRs. This may help in designing specific inhibitors.

#### Publications:

Louis JM, Wondrak E, Copeland T, Smith CAD, Mora PT, Oroszlan S. Chemical synthesis and expression of the HIV-1 protease gene in E. coli, *Biochem Biophys Res Comm* 1989;159:87.

Louis JM, Wondrak EM, Smith CAD, Mora PT, Oroszlan S. E. coli expressed HIV-1 protease mutants containing Arg87/Asn88 substitutions are inactive. In: Wimmer E, Oroszlan S, eds. *Viral proteinases as targets for chemotherapy*. New York: Cold Spring Harbor Laboratory, 1989; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05526-21 OD

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## 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)

P.I. Peter T. Mora Supervisory Chemist OD DCBD NCI

Others: C.A. Dale Smith Visiting Associate OD DCBD NCI

John M. Louis Staff Fellow OD DCBD NCI

V.W. McFarland Chemist OD DCBD NCI

## COOPERATING UNITS (if any)

Frank Hetrick, University of Maryland; Anton Jetten, National Institute of Environmental Health Sciences; K. Chandrasekaran, NIA, NIH; Vasantha Subramanian, Centre for Cellular &amp; Molecular Biology, Hyderabad, India

## LAB/BRANCH

OD, DCBD

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.75

## PROFESSIONAL:

1.75

## OTHER:

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.)

p53 full length cDNA clones were obtained from 312 Balb/c mouse cells in which the p53 is not in complex with the SV40 T antigen. Upon sequencing these p53 genes mutations were detected. The inability of these p53 gene products to complex with the SV40 T antigen is apparently due to these mutations.

The current turn-around in the suggestions on the possible role of p53 in tumorigenesis (is p53 an oncogene or an antioncogene?) is being discussed critically in the context of our experimental findings during the past several years. The role of p53 in tumorigenesis, after ten years, is still only hypothetical.

We have found a consistent correlation of p53 mRNA and protein expression with embryonal differentiation which was retained during evolution of the vertebrates. We now extended such type of observations to the induced differentiation of the mouse embryonal carcinoma cell line PCC4 in tissue culture to fibroblastic cells and to adipocytes. In the same system very large changes (increases) were observed in the mRNA for c-abl and c-fos oncogenes upon the induced differentiations. The c-abl gene has homology to the tyrosine kinase gene and we are exploring the possible role of this induction in differentiation by transfection experiments.

The experimental work has been terminated on p53 in this laboratory.

Major Findings:1. Complexing of the SV40 large T Ag with p53

We found earlier (Virology 155:132-147,1986) that in a whole family of Balb/c 3T12 cells, the p53, after SV40 transformation, is not in complex with the SV40T antigen. We cloned out p53 cDNA from two such cells, 3T12-3 a Balb/c embryo mass cell line, and 3T12-TC a tumor cell line which was reestablished from a tumor induced by the Balb 3T12 mass line and then grown out from a single cell clone. We sequenced the two full length cDNA p53 clones (work with K. Chandrasekaran) and found that the 3T12-3 p53 had a mutation at Ala 135 -> Val, and the 3T12 TC had a mutation at Tyr 233 -> Asp, as compared to other mouse p53 molecules, including a putative wild type mouse p53. (A p53 cDNA clone obtained from the F9 embryonal carcinoma cell was designated by some investigators recently as wild type on the basis of studies on mutation which are required to activate the p53 gene for cooperation with the ras oncogene for transformation for cell growth in culture.) The full length cDNA clones isolated by us are the first natural mutants where the inability of complexing with T antigen seem to be caused by mutations. Now numerous such non complexing p53 molecules (mutants?) have been observed.

In 1986, the small group of scientists engaged in p53 research received our results on the non complexing of the T antigen and p53 skeptically, as the interaction with the SV40 T antigen was thought to be general. Furthermore, then the various speculation on the possible function of the p53 centered around how the transformation of a cell occurs by p53 (which was thought to be a dominant nuclear oncogene), and how in case of complexing with the T antigen the p53 effective concentration increases by the stabilization of the 53, (which in the free form has indeed a short half life). Also, numerous papers were published claiming that p53 occurs either in larger quantity or possesses a longer half life in tumor cells than in normal cells. These studies were, however, inadequate to make such a conclusion as they lacked controls: normal cells on which the comparisons can be based.

The p53 was first detected in my laboratory in SV40 transformed mouse cells and was brought to the attention of virologists. However, my laboratory detected p53 in large amounts only in SV40 transformed cells, and we observed no discernable difference in quantities in normal cells, or in non-SV40 transformed but highly tumorigenic spontaneously transformed derivative cells derived from the normal cells. We published this in 1979. In 1982 my group published a paper in which we carefully compared the amount and the half life of p53 in two sets of cells derived from cloned normal non-tumorigenic cells, but also containing direct derivative cloned cells with very high tumorigenicity; there were no differences in p53, and the half life was uniformly short (<30 min). In 1986 we confirmed these by measuring steady state levels of p53 in Western blots using the monoclonal antibodies which became available and published such findings. For these reasons I remained critical of the designation of the p53 as an oncogene (acting in dominant fashion). However, such a designation became accepted generally after the publication in 1984 that the p53 gene cooperates with the ras gene in transformation of cells upon co-transfection, thought to be a hallmark

of the nuclear oncogenes. Recently, a paper was published claiming that mutations are required to activate the p53 gene for cooperation with the ras oncogene and for transformation (Hinds et al., J Virology 1989). By this work the "wild type" p53 was designated as represented by the cDNA clone from the F9 embryonal carcinoma cells, which alone out of numerous cDNA and genomic p53 clones activates the p53 for this cooperation with the ras oncogene. It is also concluded by the authors who carried out the transfection experiments (and until now, used as the crucial argument to support the dominant oncogene theory) that inadvertently all of their previous experiments used mutant p53 genes for the transfection experiments. Now a new theory was proposed. This theory is based on the observation that the transforming potential of p53 seems to be activated by a wide variety of mutations throughout the protein, suggesting that the activation of the p53 transforming potential is due to inactivation of a wild type p53 activity. This new theory proposes that the p53 mutants function in transformation by acting as dominant inhibitory proteins, that block the activity of the wild type endogenous p53 which should be designated as a recessive anti-oncogene (similar to the retinoblastoma gene). Recently two colorectal carcinomas were analyzed (Baker et al., Science 244:217-221,1989) and in both cases mutations were detected in the p53, and a possible suppressor gene activity of the wild type p53 was also postulated. As both the retinoblastoma gene product and p53 interact with the large T antigen of SV40, it is indeed possible that the large T-antigen functions in SV40 transformed cells as an oncogene because the binding of its gene product inactivates the suppressor function of the Rb protein (or p53). (Similarly, the adenovirus E1A may contribute to viral oncogenesis in adenovirus 5 transformed cells by binding the Rb protein [and p53]; the binding of p53 occurs, however, solely with the adeno 5 E1A). These are interesting suggestions on the Rb gene which still have to be proved. As far as p53, unlike in the case of the Rb gene, of course, this is pure speculation. There were no genetic inheritance studies done in families to suggest the role of p53 in a specific cancer. The analogy, based on T antigen binding may be only formal and not reflective of function. However, it is indeed ironical that at least two formerly very active proponents of the dominant oncogene theory of p53 turned completely around, and now advocate that p53 is an anti-oncogene.

The work in this laboratory on the non complexing of p53 from Balb/c cells, summarized in the first paragraph of this section, was initiated more than two years ago to clarify the questions of p53 complexing to T antigen and in transfection experiments the possible role of p53 in cellular transformation to tumorigenicity. The mutations we observed, the non complexing with the T antigen, the absence of recognition of an epitope by the anti-p53 conformation dependent monoclonal antibody pab-246, all can be considered now by the changed outlook as acceptable and fitting to the new theories, and the non complexing of many cellular (mutant) p53, first observed in our laboratory, are now rapidly confirmed in many cells. We have not yet carried out the transfection experiments as all of our laboratory experiments in the last six months have been on the protease of the HIV-1 virus. However, we are planning to complete these transfection experiments through our collaborators at laboratories elsewhere.



In summary, the proposal that p53 has a role in tumorigenesis, by any mechanism, remains as yet unproven. Further work is necessary to clarify what is the biological function of p53.

## 2. Differentiation and expression of oncogenes

a) P53 in chicken embryogenesis: We have observed a general decline during embryonal development of both mouse and chicken in the steady state levels of the p53 protein, and an equal decline of p53 mRNA, as measured by Western and Northern blots. From various other measurements, including nuclear run-on analysis of p53 mRNA, half life determinations, etc., we concluded that (i) the down-modulation of p53 mRNA (and of protein) during embryonal development has been conserved during the evolution of the vertebrate. This implies, but certainly does not prove, that the p53 protein may have a function in embryonal development. A sequence homologous to the mammalian p53 in fish cell has been detected. This opens the way for possible extension on the evolutionary scale the above observations on mammals and birds to fish. These experiments, with F. Hetrick, were also put on hold, to concentrate on the HIV-1 protease.

b) Changes in various oncogene expressions in induced differentiation in cell culture: In a collaborative work with A. Jetten (NIEHS) the induced differentiation of the PCC4 embryonal carcinoma cell line was studied. Upon addition of retinoic acid the PCC4 cells became in four days distinctly fibroblastic, and increased their doubling time from 12 to 24 hrs. These fibroblastic cells (denoted as PCD cells) can be further differentiated by the action of the glucocorticoid hormone dexamethason, when they become contact inhibited (within one day) in crowded cultures and they stop dividing and do not synthesize thymidine. After 4-5 days 80-90% of these cells then become adipocytes. The amount of p53 mRNA declines 70% during this last differentiation to adipocyte cells. We have also measured the mRNA of numerous proto oncogenes. Significant changes occurred in the level of the expression of abl i.e., a 30x increase rapidly (in 6 hrs) upon addition of the retinoic acid (these very high levels of the abl remained throughout the rest of the differentiation to PCD and adipocyte cells) and also an 8x increase in 824 hrs in c-fos, which remained elevated (5x) in the adipocytes. Scr expression declined 80% in the PCD cells, and became almost undetectable (0.3%) in the adipocytes, Ha ras, K-ras, and raf expression declined in PCD and adipocyte cells 70-80%. The possible significance of all these changes is being further evaluated in Dr. Jetten's laboratory. The induced differentiation of the PCC4 cells, when at their stage of PCD cells they stop dividing but continue to differentiate to adipocytes, allows the segregation of the observed effects in differentiation (such as the decline in p53 levels) from that which may occur because of changes in cell division. Much of this work is being prepared for publication. All experimental work was also suspended in our laboratory during the past six months.

The c-abl gene has homology to the tyrosine kinase gene, and we plan to explore the possible role of the above observed very rapid and large induction in cellular differentiation by transfection experiments in Dr. Jetten's laboratory.



The interesting finding on the  $\beta$ -adrenergic regulation of c-fos gene expression in an epithelial cell line is being further worked on by E. Kousvelari at the NIDR.

Searching for proto oncogenes in established fish cell lines is also being brought to a conclusion. This work is now being carried out essentially in other laboratories (LTCCB, NCI, and by F. Hetrick at the University of Maryland). Plans are being developed for further work, at the University of Maryland, possibly tying this work to the appearance of tumors in fish.

Experimental work in this laboratory is terminated on all of the above projects.

Publications:

Louis JM, McFarland VW, May P, Mora PT. The phosphoprotein p53 is down-regulated post-transcriptionally during embryogenesis in vertebrates, *Biochim Biophys Acta* 1988;950:395-402.

Kousvelari E, Louis JM, Thang L, Curran T. Regulation of proto-oncogenes in rat parotid acinar cells in vitro after stimulation of  $\beta$ -adrenergic receptors, *Exp Cell Res* 1988;179:194-203.

Yeh C-K, Louis JM, Kousvelari EE.  $\beta$ -adrenergic regulation of c-fos gene expression in an epithelial cell line, *FEBS* 1988;240:118-122.

Smith CAD, Louis JM, Hetrick FM. Presence and transcription of the p53 oncogene in the EPC cell line, *J Fish Dis* 1989;11:525-530.

Mora PT, Winterbourne DJ, Smith CAD. A clonal analysis of malignancy, *Bioscience Rep* 1989, in press.

Read-Connole B, Smith CAD, Hetrick F. Sequence homologous to mammalian proto-oncogenes and their expression in fish cell lines, *J Aquat Animal Health* 1989, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Regulation of Immune Response to Tumor Cells and Alloantigens

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

PI: C.C. Ting Senior Investigator OD DCBD NCI

Others: M.E. Hargrove Microbiologist OD DCBD NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Office of the Director, DCBD

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## 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.)

1. In vivo anti-tumor activity of CD3-AK cells. (A). CD3-AK cells were induced by activation of mouse splenocytes with  $\alpha$ CD3-AK antibody. The conventional LAK cells were induced by activation of mouse splenocytes with purified human recombinant IL-2. We found that different tumors showed different degrees of susceptibility to the in vivo killing by CD3-AK or LAK cells and the results also correlated with the in vivo effect of the killer cells on these tumors. A plasmacytoma P815 was much more susceptible to the killing by CD3-AK than by LAK cells, and the in vivo growth of P815 cells was inhibited by simultaneous inoculation of CD3-AK and was not affected by LAK cells. In contrast, a melanoma JBMS was susceptible to the killing by LAK but was resistant to CD3-AK mediated killing, and the in vivo growth of JBMS was retarded by LAK cells and was not affected by CD3-AK. Due to the ability of CD3-AK to expand rapidly in vitro, CD3-AK may be an attractive candidate for immunotherapy of susceptible tumors.

2. Mechanisms for lymphocyte-mediated cytolysis. (B). Two subsets of killer cells were induced by  $\alpha$ CD3. The killer cells that were maintained in IL2 and  $\alpha$ CD3 (CD3-AK<sup>+</sup>) induced fast lysis, whereas the killer cells that were maintained in IL-2 alone induced slow lysis. Protein kinase C (PKC) was not required in fast lysis but was required in slow lysis. Cytolytic granules might be involved in fast lysis but had very little effect in slow lysis. On the other hand, cytokines such as IL-2, IL-4 and TNF were involved in slow lysis but had no effect in fast lysis.

Major Findings:I. Tumor Immunobiology: In Vivo Antitumor Activity of CD3-AK and LAK Cells.

CD3-AK cells were induced by activation of mouse splenocytes with  $\alpha$ CD3 antibody. LAK cells were induced by activation of mouse splenocytes with purified human recombinant IL-2. We found that different tumors showed different degrees of susceptibility to the in vitro killing or in vivo anti-tumor activity of CD3-AK or LAK cells. The mastocytoma P815 was much more susceptible to the killing by CD3-AK than by LAK cells whereas a melanoma JBMS was more susceptible to the killing by LAK than by CD3-AK cells. Testing by the in vivo tumor neutralization experiment, the growth of P815 cells was inhibited by CD3-AK and was not affected by LAK cells. In contrast, the growth rate of JBMS was retarded by LAK cells and was not affected by CD3-AK. We are recently testing the in vivo antitumor activity of CD-3-AK cells on a methylcholanthrene-induced sarcoma T-92 which was susceptible to the killing by both CD3-AK and LAK cells. Our data obtained so far showed a good correlation between the in vitro and in vivo anti-tumor activities of CD3-AK and LAK cells. Due to the fact that CD3-AK can be rapidly expanded in vitro in a relatively short period of time (a 100 fold increase in 1 week), the CD3-AK cells may be an ideal candidate for the use in immunotherapy of susceptible tumors.

II. Regulation and Control of Immune Response.

## 1. Mechanisms for lymphocyte-mediated cytotoxicity.

Our understanding of the mechanisms for lymphocyte-mediated cytotoxicity has been facilitated by the rapid progress in molecular immunology and availability of T cell clones in large quantity. The demonstration of cytotoxic granules involved in lymphocyte-mediated cytotoxic reactions has aroused considerable interest in this field. Most of the studies dealt with cytotoxic lymphocytes that mediate fast lysis which was demonstrated by a short-term 4-6h  $^{51}\text{Cr}$  release assay. There were other sets of cytotoxic lymphocytes that mediated slow lysis, which required 16-20h to complete the lytic reactions. During our studies on the characterization of CD3-AK cells, we found that CD3-AK cells that were cultured in the presence of  $\alpha$ CD3 ( $\text{CD3-AK}^+$ ) mediated fast lysis, and CD3-AK cells that were cultured in the absence of  $\alpha$ CD3 ( $\text{CD3-AK}^-$ ) mediated slow lysis. It was further shown that direct activation of T cell receptor (TCR) complex resulted in fast lysis and T cell activation signal which by-passed the TCR (e.g., PMA) could induce slow lysis. In activation phase protein kinase C (PKC) was required for the activation of the activated killer cells that activated fast lysis or slow lysis. However, in lytic reactions, fast lysis was PKC independent, whereas slow lysis was PKC dependent. These findings suggested that in slow lysis, an activation phase preceded the lytic phase. We found that different cytokines might be involved in slow lysis, whereas cytotoxic granules only involved in fast lysis. Therefore, different pathways might be involved in lymphocyte-mediated cytotoxicity.

2. Requirement of PKC in the activation of killer cells.

The requirement of PKC varies in the activation of alloreactive CTL, CD3-AK and LAK cells. For allosensitization, both proliferative response (PR) and cytotoxic response (CR) were strictly PKC dependent. For  $\alpha$ CD3 activation, P.R was partially PKC dependent and CR was largely PKC dependent. For activation by IL-2 both P.R and CR were PKC independent. It appeared that PKC was required when the activation process involved T cell receptor (e.g., allosensitization), and PKC was less critical when TCR was not directly involved (e.g., IL-2 activation).

3. The role of anti-suppressor cells in the generation of LAK cells.

We previously showed that anti-suppressor T cells could be induced by allosensitization. However, we failed to show the existence of antisuppressor cells during LAK cell generation.

Proposed Course of Research:

1. In vivo antitumor activity of short-term and long-term cultured CD3-AK cells, and comparison with the effect of conventional LAK cells. (A).
2. Mechanisms of lymphocyte-mediated cytolysis. To determine the role of protein kinase C, cytokines and cytotoxic factors in fast lysis and slow lysis. (B).
3. Requirement of protein kinase C in the generation of antigen-specific and antigen-nonspecific response. (B).

Publication:

Yun YS, Hargrove ME, and Ting CC. In vivo antitumor activity of the anti-CD3-induced activated killer cells. Cancer Res 1989; in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00941-33 OD

## PERIOD COVERED

October 1, 1988 to September 30, 1989

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 Mice

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

P.I. Delta E. Uphoff

Research Biologist

OD DCBD NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

OD DCBD

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1 1/2

## PROFESSIONAL:

1

## OTHER:

1/2

## CHECK APPROPRIATE BOXES)

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

B

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

Successful marrow replacement requires elimination of normal or neoplastic hematopoietic cells without destroying other vital organs, as well as having available a genetically compatible donor. Since the first successful marrow transplantation experiments in 1950, there have been problems involving the reproducibility of the experimental data. Beyond the genetic factors involved in selecting marrow donors, little attention has been paid to the many other factors influencing successful marrow replacement. The disseminated nature of this organ system must be considered when using irradiation in preparation for engraftment. Contrary to popular belief hematopoietic cells are subject to significant repair potential with the greatest benefit obtained with simultaneous dorsal-ventral (dual) exposure at a high exposure rate of 126R/min. Significant reduction in effective purging of the system occurred with unidirectional exposures at half the exposure rate. In addition, physicists recommend the defining of the quality of x-rays in terms of half value layer since biological systems are supposedly lacking the sensitivity to detect differences on the quality of x-rays. However, the quality of x-rays does affect the outcome of the experiments when attempting permanent marrow engraftment. The exact nature of this effect has not yet been defined. Another question that requires consideration is whether marrow transplantation is a uniquely sensitive system or are these fallacies perpetuated by a failure to conduct biological experiments with the precision required to detect these critical phenomena.

Major Findings:

While genetic screening for compatible tissue donors has increased successful transplantation of many organs, marrow replacement has been an exception. Even with donor selection, cytotoxic drugs may be required for preparation of the recipient and for control of the immune response after transplantation. Unlike other organs, the hematopoietic system is unique consisting of circulating cells, cells of soft-tissue organs and cells encased in skeletal bone which must all be destroyed before permanent marrow replacement can be successful. In addition, the treatment may fail for reasons other than the method of preparing the recipient for the marrow graft, e.g., back in 1958 (Proc AACR) we reported that high leukemic mice receiving compatible marrow grafts from low leukemic donors may develop leukemia in the grafted marrow: whether relapse in leukemic patients represents a failure to destroy all neoplastic cells or an induction of leukemia in the grafted marrow is unknown. There were two other observations that made it apparent that too little attention was paid to physical factors required for successful marrow replacement. We were unable to duplicate our own data with a new x-ray machine using identical filters and adjusted to the same half value layer (HVL). Admittedly physicists consider the HVL a crude measure of the quality of x-rays but it has been considered adequate for use with biological materials. In addition dual and dorsal exposures that were equally lethal produced different survival patterns when combined with marrow grafts. Reversal and mixed chimerism were less than 5% following dual exposure but were more common following dorsal exposure. Testing the effectiveness of the radiation treatments involved progressively increased exposures without benefit of marrow grafts. Contrary to popular belief, hematopoietic cells were subject to exposure-rate effects and undergo significant repair with reduced exposure rates e.g., 63R/min was less effective than 126R/min for x-ray. Although the uniform distribution of radiation through the target was important, the benefit of the simultaneous dorsal-ventral (dual) exposure was an exposure rate effect since dual and dorsal exposures at 63R/min produced identical results. Ventral and alternating exposures were least effective for, in addition to the exposure-rate, there was a lack of homogeneity reducing exposure to critical organs distal to the radiation source. Success of alternate exposure was a function of the direction of the first exposure which indicated that initiation of repair occurred only during the exposure and required at least 11 minutes to become established with the completion of repair requiring a much longer period. These results were used for comparison with gamma radiation from CS 137 since it was not practical to compare dual and dorsal exposure at the same exposure rate as was done with x-rays. The higher energy of gamma radiation resulted in greater penetration and less absorption which required an increased exposure compared with x-rays. Using a modified gamma-cell-40 cesium irradiator, dual exposures were most effective and in decreasing order of effectiveness were dorsal, dorsal + ventral, ventral + dorsal and ventral exposures. This was the exact pattern established for x-rays. By further subdividing alternate exposures into four equal fractions the effectiveness was similar to that of the ventral + dorsal exposure. Why a dorsal + ventral + dorsal + ventral exposure was reduced to the effectiveness of a ventral + dorsal exposure was not apparent. However,

for both x-rays and gamma ray the greatest biological effect at the lowest exposure occurred with dual irradiation, a phenomenon first described for the treatment of neck tumors (Am J Roentgenol 1938). In all other treatment combinations the exposure had to be increased to obtain equivalent biological effectiveness. Increasing homogeneity without increasing exposure rate should not be expected to increase the effectiveness of the treatment.

The final problem yet to be resolved is what change in the quality of x-rays, not detected by the HVL measurement, is so readily detected when mice were prepared for marrow engraftment with variables limited to the two x-ray machines or the presence or absence of an auxillary line voltage regulator.

Publications:

None

## CONTRACT RESEARCH SUMMARY

Title: Facility for Preparing and Housing Virus Infected Mice, Genetically Manipulated Mice, and Chimeric Mice

Principal Investigator: Mr. Brian Weatherly  
Performing Organization: Bioqual, Inc.  
City and State: Rockville, MD

Contract Number: N01-CB-8-5608

Starting Date: 9/30/88

Expiration Date: 9/29/93

Goal: Perform a variety of in vivo experiments in mice (up to a colony of 3600 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 Experimental 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  $\gamma$ -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: A number of experiments involving virus infection, lymphocyte transfer, and combinations of the above have been performed in several mouse strains to investigate the synergistic effects of cytomegalovirus and graft-vs-host reaction. Approximately 6200 mice were received into the contract facility during the year, and the facility contained between 2800 and 3000 mice at any given time. Two preparations each of cytomegalovirus and influenza virus were made. Approximately 870 radiation chimeras, 4000 i.p. inoculations, 1000 i.v. injections, 400 tail bleeds, 200 thymus grafts, 30 virus plaque assays, 150 skin grafts, 600 embryos were transplanted into pseudopregnant mice, 80 lymphocyte preparations, 240 tumor palpations, and 400 ELISA tests for cytomegalovirus antibodies were performed during FY 1989. The mice have been delivered to the Experimental Immunology Branch laboratories as requested. Although other aspects of the contract are progressing, the embryo transfer and transgenic part of the contract continues to be a problem, with little or no evidence of progress.

Significance to Cancer Research: This experimental mouse facility is required to support the intramural research programs of the Experimental 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, genetic manipulation of hemopoietic cells, immunological resistance to syngeneic tumor development, models of immune deficiency, and reconstitution of the immune system.

Project Officer: Dr. Gene Shearer

Program: Immunology Resource

Technical Review Groups: Intramural Support Contract Subcommittee A

FY 89 Funds: \$434,640 EST.



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: N01-CB-85607  
Starting Date: 11/1/87      Expiration Date: 10/31/93

Goal: Maintain colonies of inbred mice (12,000 animals), inbred rats (500 animals), and rabbits (40 animals) and carry out selected breeding protocols with these animals as specified by the project officer. These animals are to be maintained in support of intramural research programs in the Immunology Branch, NCI.

Approach: Colonies of mice, rats, and rabbits are to be housed and fed according to National Research Council standards. Technical manipulations and breeding are to be carried out as directed by the project officer.

Progress: This contract represents predominantly a facility for holding experimental animals in support of the research of the Immunology Branch. As such, the contractor has maintained a colony of 12,000 mice, 500 rats, and 40 rabbits. Breedings of certain strains of mice and rats for experimental needs of the Branch have also been performed when such animals have not been available commercially. Frozen samples of sera and cells are stored in freezers of appropriate temperatures and are transferred to NIH as required.

Performance on this contract has been highly satisfactory. The animal colonies have been established and are being maintained according to National Research Council standards. Animal health has, in general, been excellent, and breeding protocols have been satisfactory. Recordkeeping and transferring of animals to and from the NIH Campus have all been satisfactory. Maintenance of frozen products in appropriate freezers has been satisfactory.

Significance to Cancer Research: This animal colony is necessary in support of intramural research programs in the Immunology Branch of NCI. Many of these programs are concerned with the immune response to cancer.

Project Officers: Dr. David H. Sachs and Dr. John R. Wunderlich  
Program: Immunology Resource  
Technical Review Group: Intramural Support Contract Subcommittee A  
FY 89 Funds: \$780,701

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: N01-CB-7-1010  
Starting Date: 6/30/87                      Expiration Date: 6/29/93

Goal: To perform radioimmunoassays of immunoglobulin molecules as well as ELISA assays of soluble interleukin-2 receptor molecules in lymphocyte culture supernatants or in biological fluids.

Approach: The contractor is to quantitate human immunoglobulins in various fluids using double antibody radioimmunoassay procedures and reagents defined and supplied by the project officer. In addition the contractor is to utilize an established ELISA assay for the soluble form of the IL-2 receptor to quantitate the level of this peptide in the serum of patients. Furthermore, the contractor is to measure antibodies produced by lymphocytes stimulated by antigens in vitro. This contract provides critically required research support for the study of primary and acquired immunodeficiencies including those associated with a high incidence of malignant transformation and with human B- and T-cell leukemias.

Progress: The contractor has established the required radioimmuno- and the ELISA assays. Elevated IL-2 receptor levels have been demonstrated in the sera of patients with HTLV-I Adult T cell Leukemia, HIV Associated AIDS, Hairy Cell B Cell Leukemia, or Hodgkin's Disease. In these patients, the serum receptor levels were indicative of tumor binding and favorable responses to therapy were associated with reductions in the serum level of Tac protein. Elevations of Tac protein in the serum were also observed in patients with select autoimmune disease and in individuals rejecting an allograft.

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. 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, assessment of prognosis, and in monitoring therapy of IL-2 receptor positive malignancies.

Project Officer: Thomas A. Waldmann, M.D.  
Program: Cancer Biology Resource  
Technical Review Group: Ad Hoc Technical Review Group  
FY: 1989              Funds: \$237,998

## CONTRACT RESEARCH SUMMARY

Title: Transplantation, Induction, and Preservation of Plasma Cell Tumors in Mice and the Maintenance of Special Strains

Principle Investigator: Judith Wax  
Performing Organization: Hazleton Laboratories  
City and State: Rockville, MD

Contract Number: N01-CB-71085  
Starting Date: 02-01-87                      Expiration Date 01-31-92

Goal: Induction, transplantation, preservation and shipping of plasmacytomas, T- and B-cell lymphomas in mice. Breeding of (congenic) strains of mice to find genes controlling susceptibility and resistance to the induction of plasma cell tumors by pristane; maintenance of wild mouse colony.

Approach: Maintain a closed conventional colony of inbred and congenic strains of mice, suitable for maintaining mice for long term plasmacytoma induction experiments. Develop BALB/c congenic strains carrying plasmacytomagenesis resistance (PCT-R) genes. Carry out procedures for identifying markers used in the construction of congenic strains. Maintain colonies of pedigreed wild ascites, tissues, high molecular weight DNA, pedigreed breeders to qualified investigators and collaborators.

Progress: Contractor has carried out basic plasmacytoma induction experiments using pristane alone in various BALB/c.DBA/2 congenic C x S RI, (BALB/c x DBA/2)<sub>F1</sub> and BALB/cAn.BALB/cJ congenic strains. In addition, contractor has carried out plasmacytoma induction experiments in pristane conditioned mice with RIM, IM, R, J<sub>2</sub>, J<sub>3</sub>, J<sub>5</sub> and other myc containing viruses. Contractor transplants essential tumors and supplies tissues and/or DNA to investigators in the Laboratory of Genetics for molecular studies. Contractor ships mice, tumors, DNA, serum or ascites to other investigators. Contractor continues to develop essential congenic strains that are being used to identify new genes. Contractor has acquired the capability to test in mouse for RFLP polymorphisms and there by advance the congenics. Through Joan Renner, Hazleton Laboratories has reorganized and revitalized the contract. In addition to the above described work, the contractor functions as the major animal resource for the Laboratory of Genetics.

Significance to Cancer Research: Provides essential support for the study of plasmacytomagenesis (carcinogenesis) with the specific goal of determining the genetic basis of susceptibility to tumor induction by mineral oil. Supplies essential biological material for investigators studying the biology of neoplastic plasma cells, tumor immunology, the genetics of immunoglobulins, and immunoglobulin synthesis.

Project Officer: Dr. Michael Potter, Dr. Beverly Mock  
Program: Immunology Support  
Technical Review Group: Intramural Support Contract Proposal Review Committee  
FY 89 Funds: \$850,769

B

## CONTRACT RESEARCH SUMMARY

Title: Maintenance and Development of Inbred and Congenic Resistant Mouse Strains

Principal Investigator: Ms. Daniella Livnat  
Performing Organization: Hazleton Laboratories America, Inc.  
City and State: Rockville, MD

Contract Number: N01-CB-71091  
Starting Date: 2/1/87                      Expiration Date: 1/31/93

Goal: To maintain a colony of inbred pedigreed strains of mice which are needed to support ongoing NCI intramural research in transplantation immunology.

Approach: The contractor maintained a colony of approximately 40 special inbred and congenic resistant strains of mice by pedigreed brother-sister mating. Quality control testing was carried out at each generation by cytotoxicity typing of breeders from each strain. Alloantisera were raised between mouse strains to assist in this quality control typing, and sera and animals were shipped by the contractor to collaborating investigators at NIH and elsewhere.

Progress: The contractor maintained all inbred and congenic resistant strains of mice in excellent condition. Breeding of each strain and of hybrid strains, recordkeeping, and quality control testing were all highly satisfactory. A backcrossing program was used for all congenic resistant strains in order to keep the backgrounds of these strains identical. This involved backcrossing of each congenic to the reference background line once every 6-10 generations. This program has also been very satisfactory to date. Fourteen new recombinant H-2 haplotypes have been identified during the process of this backcrossing, and these have been bred to homozygosity and established as new valuable inbred congenic strains.

In addition, fusions for hybridoma production were performed by the contractor, with the purpose of developing monoclonal antibodies to histocompatibility antigens. Antisera for histocompatibility antigen typing were prepared in various combinations and were found to be excellent reagents. A series of strain-restricted typing sera were produced in order to distinguish each strain in the colony from all other strains. Shipping of animals and sera to collaborating investigators at NIH and elsewhere was satisfactory. The animals shipped were generally of excellent health and provided breeding stock for the production of experimental animals in numerous laboratories. Computerization of records and reports has been performed and has led to efficient data handling. Hybridoma reagents were produced, stored and shipped starting with cell lines developed by the Project Officer.

Significance to Cancer Research: This animal facility was needed for the breeding and maintenance of these inbred congenic resistant strains of mice. These animals made possible research on individual histocompatibility antigens and, in particular, the role of the major histocompatibility complex in the transplantation of tissues and cells and in the immune response to cancer.

Project Officer: Dr. David H. Sachs  
Program: Immunology Resource  
Technical Review Group: Intramural Support Contract Proposal Review Committee  
FY 89 Funds: \$590,000

B



Title: Feral Mouse Breeding Colony

Principal Investigator: Dr. Joan Renner and Ms. Evelyn Hogg  
Performing Organization: Hazelton Laboratories America, Inc.  
City and State: Rockville, MD

Contract Number: N01-CB-95621  
Starting Date: 12/01/88 Expiration Date: 11/30/91

Goal: Induction of mammary tumors with biological (hormones and mouse mammary tumor virus, MMTV) and chemical carcinogens in various feral strains of Mus musculus and other species of Mus. Breeding of congenic strains of feral M. musculus that contain specific genetically transmitted MMTV genomes.

Approach: Maintain a closed pedigreed colony of 1,000 feral and inbred mice. The colony is composed of approximately 700 mice that are held long-term (2 years) for tumor development and 300 mice as a breed nucleus. The breeding nucleus is composed of four pedigreed outbred colonies of feral mice having unique characteristics that are pertinent to the study of mouse mammary tumorigenesis. They are: Czech II V<sup>+</sup> mice (Mus musculus musculus), Czech II V<sup>-</sup> mice, TAK (M. cervicolor popaeus) mice, and MS (M. spretus) mice. In addition, a limited breeding nucleus of the high-incidence C3H/OuJ and GR inbred mouse strains and the low-incidence BALB/c  $\pi$  inbred strains are maintained. BALB/c mice foster nursed on MS, Czech II V<sup>+</sup>, and MCPT are also maintained for development of mammary tumors.

Progress: The contractor maintains the feral mouse colony in excellent condition, and carries out the breeding program, quality control, and maintenance of records in a highly satisfactory manner. Approximately 30 or more mammary tumors were obtained from each of the BALB/cfC3H, CZECHII V<sup>-</sup> fC3H, C3H, GR, CZECHII mouse strains. Other sublines of BALB/c mice foster-nursed on M. spretus, M. cervicolor propaeus tak, and CZECHII V<sup>+</sup> have been developed. MTV-1 and MTV-2 have been introduced into the CZECHII V<sup>-</sup> genetic background and both are at backcross N9. Twelve mammary hyperplastic outgrowth lines from CZECHII V<sup>+</sup> mice are being carried to determine the frequency of mammary tumor development. Single cell suspensions of primary mammary epithelium have been introduced into cleared mammary gland fat pads and shown to repopulate the gland.

Significance to Cancer Research: Provide essential support for the study of mammary tumorigenesis with the specific goal of identifying and characterizing the genes at risk to MMTV activation. Provides essential biological material for other investigators studying the biology of the mouse mammary tumor virus as well as other classes of retroviral genomes.

Project Officer: Dr. Robert Callahan  
Program: Immunology Resource  
Technical Review Group: DEA; Ad Hoc Intramural Technical Review Group

FY 89 Funds: \$144,582.



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