

Division Of

**Cancer Biology,  
Diagnosis, and  
Centers**

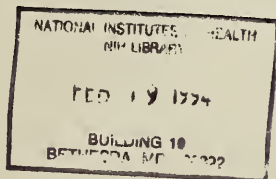
NATIONAL  
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93 annual report

**Division Of**

# **Cancer Biology, Diagnosis, and Centers**



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NATIONAL CANCER INSTITUTE  
DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS

ANNUAL REPORT

October 1, 1992 through September 30, 1993

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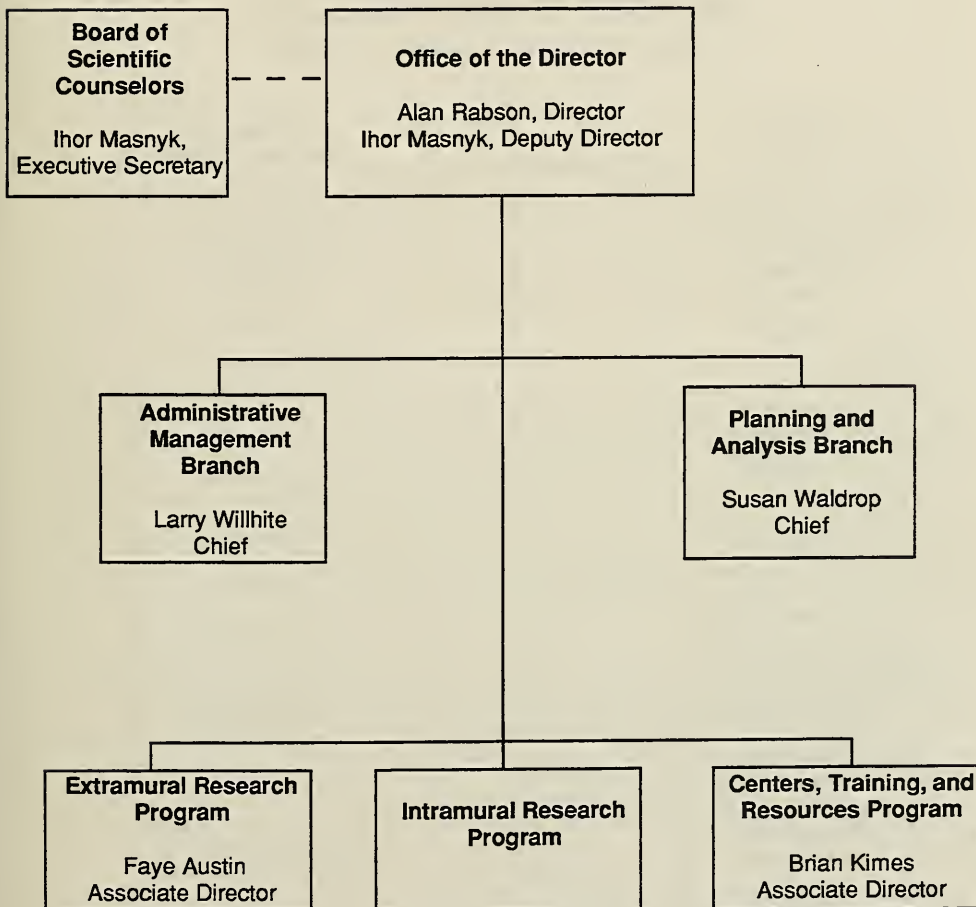
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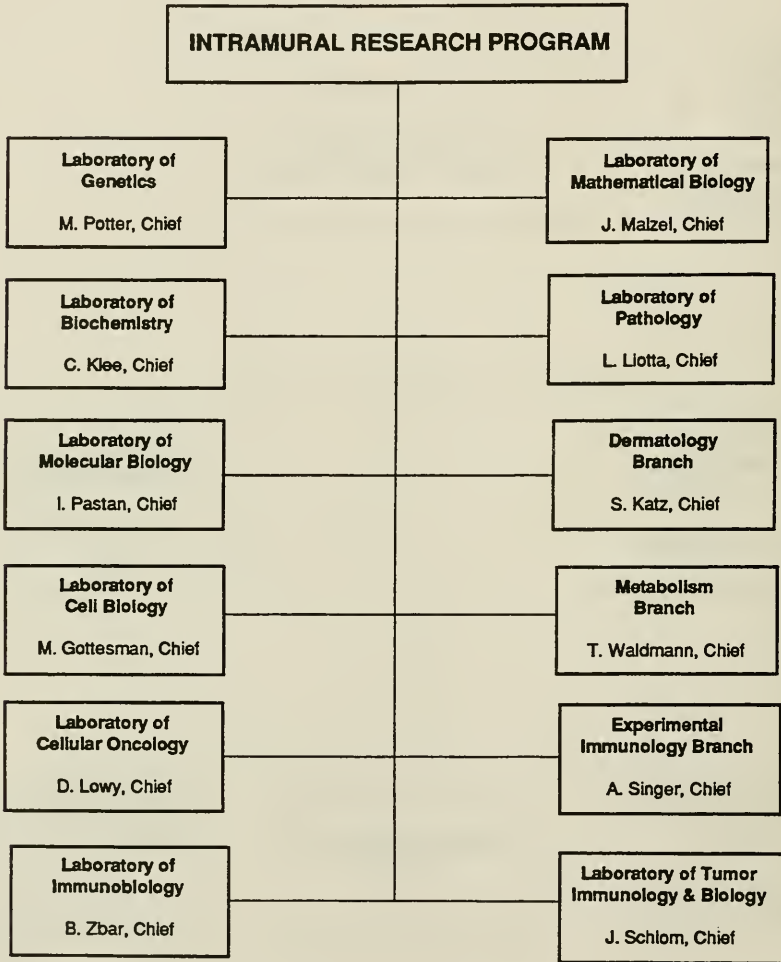




# DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS ORGANIZATIONAL CHART



**DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS  
INTRAMURAL RESEARCH PROGRAM  
ORGANIZATIONAL CHART**



NATIONAL CANCER INSTITUTE  
DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS

INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1992 through September 30, 1993

INTRODUCTION

The mission of the Division of Cancer Biology, Diagnosis, and Centers (DCBDC) in the National Cancer Institute is to conduct laboratory and clinical investigations in cancer biology, immunology, and diagnosis, and to manage the centers, training and construction programs of the NCI. These activities are carried out in the intramural laboratories and branches and extramurally through the Extramural Research Program and the Centers, Training, and Resources Program. This report describes the intramural research investigations of DCBDC.

The Intramural Research Program in the Division of Cancer Biology, Diagnosis, and Centers consists of twelve laboratories and branches at the NIH Bethesda campus and at the Frederick Cancer Research and Development Center. They are the Laboratory of Genetics, the Laboratory of Biochemistry, the Laboratory of Molecular Biology, the Laboratory of Cell Biology, the Laboratory of Cellular Oncology, the Laboratory of Immunobiology, the Laboratory of Mathematical Biology, the Laboratory of Pathology, the Dermatology Branch, the Metabolism Branch, the Experimental Immunology Branch, and the Laboratory of Tumor Immunology and Biology. Each of these laboratories is directed by a scientist of international stature, three of whom are members of the National Academy of Science and two of whom are members of the Institute of Medicine.

Research conducted in the intramural laboratories covers a broad range of investigations from the regulation of gene expression in bacteria to clinical studies of human cancer. A common theme of many of these studies is to understand the molecular mechanisms involved in the regulation of normal cell growth and differentiation, and further, to define the genetic changes responsible for neoplastic transformation and for the progression of cancer from localized to metastatic disease. While the primary focus is on basic research, considerable emphasis is placed on fostering the translation of fundamental discoveries in cancer biology and immunology into new approaches for cancer prevention, diagnosis, and treatment.

During the past year, particular emphasis has been placed on basic research projects focused on breast cancer. Recent advances in the identification of genetic markers associated with breast and other cancers are already proving useful. For example, intramural scientists have identified NM23, a new suppressor gene associated with metastasis. They have shown that loss of the

NM23 protein is associated with a poor prognosis and poor survival in breast cancer, and that may be useful in identifying those women with node negative disease who could benefit from aggressive adjuvant chemotherapy. Considerable progress has been made in elucidating the molecular genetic mechanisms that result in tumor cell invasion. Designing new agents that can prevent the development of metastasis is a major research goal within the division. Intramural scientists have identified an agent, termed CAI, that blocks signal transduction and interferes with tumor cell motility. CAI shows particular promise for the prevention and treatment of breast and ovarian cancer. It is currently being evaluated in a Phase I therapy trial and is under development as a chemopreventive agent. Studies of basic cell biology and advances in molecular genetic technology have led to the development of a new class of cancer therapeutic agents in which cell recognition molecules (such as monoclonal antibodies and growth factors) are fused to genetically modified forms of a bacterial toxin, or are chelated to radionuclides to produce molecules capable of binding to and killing tumor cells. Preclinical studies are in progress to develop reagents based on this approach for the improved diagnosis and treatment of breast cancer. Advances in our understanding of the development of multidrug resistance in certain common tumors, such as breast cancer, and the development of techniques of gene insertion have suggested entirely new ways of overcoming the bone marrow toxicity associated with high dose chemotherapy.

A broad-based program in fundamental immunology explores the complex mechanisms governing the regulation of the immune response. The goal of these studies is to identify those aspects of the biology of tumor cells that can be enhanced to promote their recognition and attack by the immune system, as well as those elements of the immune system that can be stimulated to provide an effective antitumor response. High priority is being placed on the development of new strategies for cancer vaccine development. One approach has been the development of tumor-associated recombinant vaccines. Initial efforts have focused on CEA, a tumor-associated antigen expressed by most gastrointestinal tumors, pancreatic tumors, many adenocarcinomas of the lung, and over 50% of breast tumors. CEA alone is only weakly immunogenic; however, recent studies have shown that the co-presentation of CEA with a strong immunogen offers a way to induce anti-CEA responses for tumor immunotherapy. The gene for CEA has been introduced into a vaccinia virus, and the resulting construct is being evaluated in a Phase I clinical trial for its ability to induce a specific immune response in patients with CEA-expressing tumors. Increased efforts are being made to apply this approach to the development of vaccines against tumors expressing other tumor-associated antigens. Another approach is to use mutant oncoproteins, such as the mutant p53 protein expressed in certain tumors, to stimulate a cytotoxic antitumor response. This approach is being vigorously pursued in a collaborative study between intramural and extramural NCI scientists. Basic studies of gene regulation and expression in papillomaviruses (PV) have suggested an entirely new approach for the development of a vaccine to prevent human PV infection which is often associated with the development of cervical cancer.

The diversity and high quality of each of these research programs reflects the varied interests and training of the investigators within the Division. The continued interaction and exchange of ideas and technical expertise among

basic scientists and scientists focused on more applied clinical research problems provides a fruitful environment for the translation of advances in basic research into new strategies for the prevention, diagnosis, and treatment of cancer.

In many aspects, the DCBDC Intramural Research Program is complementary to the extramural research programs in cancer biology, cancer immunology, and cancer diagnosis. In addition, it is frequently interactive with other extramural programs, since many of the intramural scientists are involved in active collaborations with their colleagues in the NCI extramural program and in the biotechnology industry.

Selected highlights of the research activities and major accomplishments of the Intramural Research Program are described in the following pages. A more detailed discussion of the research activities of each Intramural Laboratory and Branch can be found in the Annual Reports that follow.

### **Laboratory of Genetics** **Dr. Michael Potter, Chief**

The primary research focus in the Laboratory of Genetics is on determining the mechanisms involved in the induction of plasma cell tumors (PCT). This includes studies on susceptibility and resistance genes, oncogenes, and other genes that influence tumor development.

The model system used by Dr. Potter's group is the induction of PCT by chronic peritoneal irritants such as pristane. In this model, tumors can only be induced in genetically susceptible strains; the inducing agents are not metabolically active; the tumors all have characteristic chromosomal translocations that activate the c-myc proto-oncogene; and the initiated tumor cells require a selective microenvironment for progression. Evidence from this system and others indicates that oncogenic mutations can develop and persist long before tumors develop. Detection of major oncogenic mutations may provide an experimental system for finding ways to eliminate or control these potentially dangerous cells before they progress to malignancy. The murine plasmacytoma model provides an important animal model for studies of human multiple myeloma.

Dr. Beverly Mock has analyzed inheritance of susceptibility to pristane-induced plasmacytomagenesis. During the past year, she has shown that the susceptibility locus/region is in the distal part of mouse Chr 4 near the markers D4Lgm3, D4Mit13, Gt-10 and Tnfr-1. This region of mouse Chr 4 is homologous with human Chr 1p.

A major new finding this year has been the development by Dr. Siegfried Janz of a sensitive PCR amplification technique for detecting the most common type of translocation in PCT T(12;15)-Sa-c-myc. Dr. Janz has shown that these translocations can be detected as early as 30 days after the injection of pristane. He has also found that chromosomal translocations occurring in the preneoplastic period can be remodeled. This assay is being further refined to

determine if specific genes influence the rate of formation of these translocations. Studies are also underway to determine the kinetics of translocation formation and the cell and tissue origin of these translocations.

Dr. Potter's group has recently identified a new potent plasmacytomagenic agent, dimethyl-polysiloxane, the principal component of the gels used in breast implants. This material in relatively small amounts induces the formation of a granulomatous tissue on mesenteric surfaces and these granulomatous lesions can progress to plasmacytomas.

## Laboratory of Biochemistry

### Dr. Claude Klee, Chief

Investigators in the Laboratory of Biochemistry address a series of independent, but related, research problems and provide the laboratory with a broad and complementary array of technical expertise in molecular biology, genetics, and protein chemistry. The new *Drosophila* genetics facility, organized by Dr. Mark Mortin, is now fully functional and utilized by five different groups planning to use genetic techniques in *Drosophila* as a strategy for the functional analysis of proteins in vivo.

Dr. Carl Wu's group has continued to focus on gene regulation and chromatin structure, with emphasis on the molecular analysis of transcription factors regulating the heat shock protein (hsp) genes. This group has made major progress in their dissection of the heat stress signal transduction pathway. Investigations on the role of chromatin structure on heat shock gene expression have continued, and the ability of the GAGA transcription factor to assemble an accessible chromatin structure on the heat shock promoter was analyzed, as well as the interactions between the heat shock transcription factor HSF and chromatin. Milligram amounts of HSF protein have been produced using bacterial and eukaryotic expression systems, and the purified protein is being used for physical studies and as a biochemical reagent for dissecting the stress signal pathway. The post-translational modifications of HSF that are induced by heat shock are being investigated by means of mass spectrometry.

Dr. Claude Klee and her colleagues have concentrated their efforts during the past year on the large scale expression in *E. coli* of the two subunits of the calmodulin-regulated protein phosphatase, calcineurin. They have demonstrated that myristoylation of the Ca<sup>2+</sup>-binding subunit of the enzyme is required for the enzymatic activity of the holoenzyme. The three-dimensional structure of this subunit, determined in collaboration with Ad Bax and J. Anglister (NIDDK), definitively identified calcineurin B as a member of the "EF-hand" Ca<sup>2+</sup>-modulated proteins. The major objective of this group is to elucidate at the molecular level the regulation of this enzyme by Ca<sup>2+</sup> and calmodulin. The recent demonstration by S. Schreiber and his colleagues (Harvard University) that calcineurin is the target of some immunosuppressive drugs emphasizes the need to elucidate the structure of calcineurin to further understand the mechanism of action of the drugs and to study the role of calcineurin in the Ca<sup>2+</sup>-mediated signal transduction pathway in T cells.

The major clinical and biological implications of the mapping of human genes and DNA sequences onto human chromosomes have long been an area of interest in the Laboratory. Several new genes have been mapped by Dr. Wesley McBride and his collaborators. They include the interleukin 2 gamma receptor gene (IL-2RG), a phosphotyrosine phosphatase gene, the plasma membrane ATPase isoform 3, and the transglutaminase genes 2 and 3. Dr. Maxine Singer and her colleagues have been concentrating on the mechanism of translation of the bicistronic mRNA of the LINE-1 human transposable element (LHs) and on the nature of the translation products. Dr. Dean Hamer has initiated a new challenging research program. His group is now studying the molecular basis for the sexual differentiation of neural structure and function in humans, *Drosophila*, and rats. Their most striking finding is a linkage between DNA markers on the X chromosome and male sexual orientation. If confirmed, this will provide the most compelling evidence to date that human sexuality is genetically influenced, and will represent a breakthrough in the study of human behavior.

**Laboratory of Molecular Biology**  
**Dr. Ira Pastan, Chief**

The Laboratory of Molecular Biology uses genetics, molecular biology, and cell biology to study gene regulation and cell behavior. A major goal is also to develop recombinant toxin molecules for the treatment and diagnosis of cancer, AIDS and other human diseases.

Dr. Ira Pastan and colleagues have developed an immunotoxin, termed LMB-1, in which monoclonal B3 (which reacts with multiple mucinous carcinomas including colon, some breast, and some ovarian tumors) is coupled to LysPE38, a genetically modified form of *Pseudomonas* exotoxin (PE). LMB-1 has been approved by the FDA and is ready to enter clinical trials. A second generation recombinant immunotoxin, LMB-7, combines the variable region of the B3 antibody with PE38. Efforts are underway to prepare material for clinical use. New mutant forms of PE have been created which can be selectively derivatized by polyethylene glycol to reduce immunogenicity and increase survival in the blood. Several of these mutations will be subcloned into LMB-7 to see if this recombinant immunotoxin retains activity and is less immunogenic. A chelate of the B3 antibody has been prepared and, when labeled with <sup>111</sup>In, will image tumors in mice. A clinical grade radioconjugate is currently being prepared. Single chain immunotoxins directed at the IL2 receptor have been made and shown to cause complete regression of tumors bearing IL2 receptors in mice. One of these, anti-Tac(Fv)-PE38, is being prepared for clinical development. A new antibody (PR1) that reacts with an antigen on normal prostate and prostate carcinomas has been isolated. The antibody is an IgM and the variable regions have been cloned and grafted to a human IgG1 constant region. The possible usefulness of this antibody for the therapy and/or diagnosis of prostate cancer is being examined. Other immunotoxins directed against the EGF receptor, the erbB2 protein, the IL6 receptor, and the IL4 receptor are also being developed.

Dr. Glenn Merlino and his co-workers are using transgenic mouse technology to address basic questions about the role of growth factors and oncogenes in the pathogenesis of cancer and other diseases. They have continued their study of transgenic mice overexpressing a human transforming growth factor  $\alpha$  (TGF $\alpha$ ) gene, and have generated transgenic mice overexpressing transforming growth factor  $\beta$  (TGF $\beta$ ) in the pregnant mammary gland. Currently, Merlino and co-workers are generating mice that possess more than one transgenic growth factor or oncogene (double transgenic mice) to assess their interactive potential.

Dr. Sheueyann Cheng and her colleagues are investigating the structure and activity of the human  $\beta 1$  thyroid hormone receptor (h-TR $\beta 1$ ). To understand the molecular basis of the thyroid hormone-dependent gene regulating activity of h-TR $\beta 1$ , the structure of the thyroid hormone binding domain (HBD) has been studied and recently has been determined.

Dr. Sankar Adhya is studying the mechanism of negative control of gene regulation at the level of transcription using the genes of D-galactose utilization in *Escherichia coli*. Dr. Susan Garges and Dr. Adhya are studying how the cyclic AMP receptor protein (CRP) activates transcription in *E. coli*.

Dr. Susan Gottesman's group has been studying the role of protein degradation in regulating gene expression and has continued with studies on the linkages between chromosome synthesis and partition of chromosomes during cell division. They have also continued to investigate the function of the Clp energy-dependent protease. The specificity of this protease in vivo seems to be dictated by the ATPase subunit.

## **Laboratory of Cell Biology**

### **Dr. Michael Gottesman, Chief**

The Laboratory of Cell Biology conducts research on the molecular basis of drug resistance in cancer cells, the molecular basis of p53 suppression of malignant transformation, the biological role and mechanism of ATP-dependent and acid proteases, the process of melanogenesis, the mechanism of antigen processing, and studies on the regulation of translation of HIV RNA.

The major research effort, to study resistance to anti-cancer drugs, involves a collaboration with Dr. Ira Pastan, Chief of the Laboratory of Molecular Biology. Cross-resistance to natural product drugs frequently results from expression of the *MDR1* gene which encodes a 170,000 dalton P-glycoprotein, an energy-dependent, efflux pump. Major questions under study include the mechanism of action of this pleiotropic transporter and the development of ways to improve cancer treatment. Analysis of this multidrug transporter has led to a model in which natural product hydrophobic drugs are removed directly from the plasma membrane. To test this model and to learn more about how energy is transduced to drive transport, P-glycoprotein has been purified. A drug-dependent ATPase activity has been demonstrated in reconstitution experiments. Retroviral vectors encoding the *MDR1* gene have been used to confer multidrug-resistance on bone marrow of mice resulting in selective advantage



of transduced bone marrow cells in vivo. Previous evidence from *MDR1* transgenic mice, and from transduction of erythroleukemia cells suggests that relatively high level expression of the *MDR1* gene in bone marrow has little or no deleterious effect on function. These experiments, combined with the ease of selection of the multidrug resistant phenotype, cell surface expression of P-glycoprotein which allows for detection and sorting of living cells, and the potential ability to "design" unique transporters functionally distinguishable from the wild-type transporter, indicate that the *MDR1* gene is a good candidate for gene therapy in humans. Since protection of human bone marrow from the toxicity of anti-cancer therapy using the *MDR1* gene should be possible, clinical trials testing the feasibility of this approach in patients with breast and ovarian cancer undergoing autologous bone marrow transplantation is under consideration.

Research supervised by Dr. Ettore Appella has utilized mass spectrometry for the structural characterization of peptides bound to Class I and Class II MHC molecules. His studies have shown that a large percent of peptides bound to Class II MHC molecules are derived from self Class I molecules. The predominance of these peptides derived from MHC-related proteins may be relevant to the etiology of autoimmune diseases. In addition, the structure of the binding site of the p7 nucleocapsid protein of HIV has been elucidated. A stem-loop sequence in HIV-1 viral RNA plays a major role in the binding of p7, and this is important to repress 35S RNA translation and facilitate the formation of a core structure essential for infectivity.

### **Laboratory of Cellular Oncology**

**Dr. Douglas Lowy, Chief**

The Laboratory of Cellular Oncology plans and conducts fundamental research on the cellular and molecular basis of neoplasia. The major areas of interest are ras-encoded proteins and an analysis of human papillomaviruses.

Dr. Lowy's group has been studying ras function by examining three proteins (GAP, NF1 and CDC25Mm) that influence the activity of Ras protein. NF1 is the gene that is mutated in patients with type 1 neurofibromatosis. NF1 possesses a GAP-like catalytic activity against Ras protein and shares significant homology with negative regulators of yeast Ras. Schwannoma cell lines from patients with neurofibromatosis have low levels of the NF1 product neurofibromin, and contain high levels of GTP-Ras. This suggests that NF1 is a tumor suppressor gene whose encoded GTPase stimulation negatively regulates Ras. Dr. Lowy's group also identified neuroblastoma and melanoma cell lines with genetic abnormalities of NF1 and reduced to absent levels of neurofibromin, suggesting that NF1 is acting as a tumor suppressor gene in these cell lines. In contrast to the schwannoma lines, the level of GTP-Ras was low in these lines and did not correlate with that of neurofibromin. These and other data suggest that NF1 might inhibit cell growth by a mechanism independent of its GTPase stimulatory activity.

There is a strong association between malignant progression of human genital lesions and certain "high risk" (HPV) types, most frequently HPV16. Drs. Lowy

and Schiller have expressed the L1 major capsid proteins of several human and animal PV types via baculovirus vectors. The L1 proteins were expressed at high levels and assembled into PV virion-like structures. They have identified two HPV16 L1 clones from primary lesions that, unlike the prototype L1 used in previous studies, efficiently assembled into particles. The self assembled bovine papillomavirus (BPV) L1 resembles intact virions capable of inducing high titer neutralizing antiserum. These results indicate that L1 has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions. Particles containing both L1 and the L2 minor capsid protein have also been generated. These types of particles might be considered as a candidate for a vaccine to prevent PV infection.

They have also developed an ELISA assay based on HPV16 L1/L2 particles, and have determined that two-thirds of women who are positive for HPV16 DNA by PCR have significant reactivity to the assembled virion proteins. Less than 10% of the sera from women negative for HPV DNA or positive for low risk HPV6 or HPV11 DNA gave positive reactions. This assay, or a similar one based on a mixture of high risk HPV particles, may aid in determining the natural history of high risk HPV infection and might be useful as an adjunct to Pap screening to identify women at risk for developing cervical cancer.

## Laboratory of Immunobiology

Dr. Berton Zbar, Chief

The genetic basis of human renal cell carcinoma has been the major focus of the research effort of the Laboratory of Immunobiology. The major accomplishment this year has been the isolation of the von Hippel-Lindau (VHL) tumor suppressor gene. The newly isolated gene also appears to play a major role in the pathogenesis of sporadic renal cell carcinoma.

The identification of the VHL tumor suppressor gene is the result of 8 years of study of the genetic basis of human renal cell carcinoma by Dr. Berton Zbar and his colleagues. The VHL gene was isolated after detailed genetic and physical mapping of the VHL region. It is evolutionarily conserved and encodes two widely expressed transcripts of about 6 and 6.5 kb. The partial sequence of the inferred gene product shows no homology to other proteins, except for an acidic repeat domain found in the procylic surface membrane glycoprotein of Trypanosoma brucei.

The three-year project supported by the National Center for Human Genome Research for the building of a high resolution genetic linkage map of human chromosome 3 has been completed. During the third year their effort was focused on the development and mapping of new highly polymorphic microsatellite probes instead of the previously studied RFLP markers. Altogether 40 new microsatellite and 150 RFLP markers were placed on the chromosome 3 map, reaching an 80 probe/Morgan overall density.

**Laboratory of Mathematical Biology**  
**Dr. Jacob Maizel, Chief**

Research in the Laboratory of Mathematical Biology (LMMB) covers a broad range of theoretical and experimental studies including molecular modelling, theoretical molecular calculations, membrane structure and function, and physiological modeling studies. Increased understanding of these biological systems provides models for aspects of malignant and other disease processes, and is enhanced through the use of advanced computing. Many of the theoretical studies are possible only through use of the supercomputing facilities at the Frederick Biomedical Supercomputing Center, FCRDC.

Computer analyses of proteins and nucleic acids are developed and implemented in conjunction with techniques of biochemistry, virology, and electron microscopy on sequences of picornaviruses, adenoviruses, and 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 vector and massively parallel supercomputers, minicomputers 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.

Work has also continued on the heterogeneous RNA structure analysis system with improvements in its graphical presentation capabilities, RNA database matching facilities, mutated structure generation and extensions to the MasPar interface. This system, in conjunction with gel shift experiments, has been used to help determine the binding site of nucleocapsid protein NCp7 of HIV-1 and the RNA structural components that determine this site. This protein is important for encapsidation of the virus genome, RNA dimerization and primer tRNA annealing in vitro. Results show that NCp7 binds to a unique RNA structure within the Psi region. Also, this structure is necessary for RNA dimerization. Dr. Bruce Shapiro's group has proposed that NCp7 binds to the RNA via a direct interaction of one zinc binding motif to a stem loop structure in one RNA molecule followed by binding of the other zinc binding motif to a motif in the other RNA molecule.

The research goals of Dr. Robert Blumenthal's laboratory are directed towards an understanding of mechanisms of membrane fusion mediated by viral spike glycoproteins. The mode of action of the envelope protein of HIV has been the focus of intense research effort. The initial steps of HIV envelope protein-mediated membrane fusion has been studied by continuous monitoring of fluorescent dyes during fusion using fluorescence spectroscopy and low light, image enhanced videomicroscopy. The combination of studies employing HIV-expressing effector cells and defined target membranes facilitates the testing of hypotheses regarding the role of different factors in adhesion and fusion. Transmission of retrovirus between cells is thought to be associated with cell membrane fusion. In this way the virus is not exposed to the extracellular

space and is hidden from the immune response. An understanding of the mechanism of viral fusion should enable the development of anti-HIV therapeutic agents.

## Laboratory of Pathology

Dr. Lance Liotta, Chief

The Laboratory of Pathology provides expertise and diagnostic services in anatomic pathology, surgical pathology, exfoliative cytology, fine needle aspiration, immunochemistry, hematopathology, and electron microscopy for the NIH Clinical Center physicians. The Laboratory also provides consultant services to the local medical community and to pathologists throughout the country and abroad. Research activities include evaluation of several tumor markers as prognostic indicators in breast, ovarian, and other tumor types.

The major research effort in the Laboratory of Pathology is on studies of genes and gene products involved in tumor cell invasion and metastasis. Expression of the metastatic phenotype depends on a balance between positive and negative regulatory gene products. Understanding the mechanism of action of these gene products has led to new strategies for prognosis and therapy. Dr. Patricia Steeg has identified the nm23 gene family. Expression of nm23 is reduced in highly metastatic human breast, hepatocellular and melanoma tumors. Recent studies indicate that nm23 protein has multiple biochemical activities, including a nucleoside diphosphate kinase activity and a cAMP regulated ATPase activity. The role of each biochemical activity in nm23 function is under investigation. As a cancer marker, nm23 may provide a new approach to predicting the metastatic aggressiveness of an individual patient's tumor. Agents which modulate nm23 expression or function, or mimic its action, may have therapeutic potential.

Dr. William Stetler-Stevenson is studying a metalloproteinase which cleaves basement membrane type IV collagen at a specific locus, and is augmented in metastatic tumors. Negative regulation of type IV collagenase may be mediated through TIMP-2, a novel human metalloproteinase inhibitor identified and cloned by Dr. Stetler-Stevenson. The complete domain structure of TIMP-2 has been determined, and the chromosomal location of TIMP-2 on 17q has been determined and confirmed. Current data support the hypothesis that TIMP-2 may function as a tumor suppressor protein by inhibiting metalloproteinase activity required for invasion. *In vivo* TIMP-2 also arrests metastasis through inhibition of angiogenesis. Specific clinical applications of TIMP-2 could include the treatment of bone metastasis and Kaposi's sarcoma.

Locomotion is another necessary component for tumor cell invasion. Dr. Mary Stracke is cloning the gene for a potent new motility stimulating cytokine, autotaxin. Anti-peptide antibodies, produced against selected autotaxin peptides, are being utilized for biochemical and histochemical studies of autotaxin. Dr. Elise Kohn has identified a new signal transduction inhibitor which blocks tumor cell cytokine stimulated growth and motility. The inhibitor, a substituted triazole termed CAI, constitutes a new approach to cancer therapy. In animal models using a variety of human tumors, oral

administration of CAI produced primary tumor and metastasis regression. Clinical phase I trials for treatment of refractory cancers began in March, 1992. Low toxicity and promising tumor responses have been seen in the first 14 patients. CAI is also being developed as a potential chemopreventive agent.

### **Dermatology Branch**

#### **Dr. Stephen Katz, Chief**

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 Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 2,000 patients are seen in consultation each year). Dr. John DiGiovanna's group has continued studies directed at skin cancer treatment and prevention. He is also actively collaborating with several groups to determine whether genetic linkage exists between certain heritable skin diseases and gene clusters.

Dr. Stephen Katz and his colleagues have continued their studies of the immunological functions of cells of the epidermis with particular emphasis on Langerhans cells (LC). During the past year Dr. Katz' group has initiated studies that assess the phenotypic and functional characteristics of epidermal Langerhans cells in the skin of patients with AIDS. In their first cohort of patients, they found that LC function at least as well as peripheral blood monocytes in the activation of T cells. They are continuing these studies and attempting to use, as responder cells, T cells from nonaffected identical twins. In addition, they are assessing the ability of HIV to infect LC from HIV-infected patients as well as normal human epidermal cells *in vitro*. These studies should provide insight into the role of skin as an initiator of inflammatory, and perhaps neoplastic, lesions in skin of HIV-infected patients.

Dr. John Stanley's laboratory studies autoantibody-mediated skin diseases in order to further elucidate the pathophysiology of these diseases and to better understand the structure and function of normal epidermis and epidermal basement membrane zones. cDNA cloning of pemphigus vulgaris antigen (PVA) indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules. PVA is most closely related to desmoglein I, which is in the cadherin supergene family, as is pemphigus foliaceus antigen. Pemphigus vulgaris patients' sera have antibodies that bind the amino-terminal extracellular domain of PVA, a region of cadherins thought to be important for their function of homophilic binding.

### **Metabolism Branch**

#### **Dr. Thomas Waldmann, Chief**

The clinical research program of the Metabolism Branch is directed toward developing rational approaches for the prevention and treatment of cancer,

primary immunodeficiency diseases and AIDS. A broad range of immunologic investigations are carried out in patients with primary and acquired immunodeficiency diseases that are associated with a high incidence of neoplasia, as well as in patients with cancer, especially leukemia.

Dr. Louis Staudt's laboratory focuses on the molecular cloning and characterization of novel lymphoid-restricted genes that regulate the development and function of lymphocytes. One lymphoid-restricted gene, Ly-GDI, encodes a protein bearing striking homology to a regulator of the ras-like G protein, rho. The cloning of Ly-GDI suggests a mechanism by which signal transduction through the ras-like GTP binding proteins can be regulated in a cell type-specific fashion. Another lymphoid-restricted gene, JAW1, encodes a transmembrane protein which resides in the endoplasmic reticulum. JAW1 has structural similarity to proteins involved in vesicle transport and fusion, suggesting that these processes can be regulated in a lymphoid-restricted fashion.

Major efforts have been placed on the development of IL-2 receptor-directed therapy. Dr. Waldmann previously identified two peptides that bind IL-2, and he proposed a multichain model for the high affinity receptor in which both IL-2R $\alpha$ - and IL-2R $\beta$ -binding proteins are associated in a receptor complex. A third component was identified by the laboratory of Dr. Kazuo Sugamura in Japan. Dr. Waldmann has recently identified a fourth component of the IL-2 receptor, a 30 Kd peptide that is modulated from the surface of activated T cells by the addition of interleukin-2.

Initially Dr. Waldmann focused his IL-2 receptor-directed therapeutic studies on patients with adult T-cell leukemia (ATL), using unmodified murine anti-Tac monoclonal antibody. There was no toxicity and several of the patients underwent a remission. However, rodent monoclonal antibodies often induce a human immune response which limits their efficacy. To circumvent this difficulty, genetically engineered "humanized" antibody variants of anti-Tac have been produced. This "humanized" anti-Tac monoclonal antibody is dramatically less immunogenic than the parent mouse monoclonal, and it manifests an additional antibody-dependent cellular cytotoxicity that is absent in the parental mouse anti-Tac. Therapeutic trials have been initiated using humanized anti-Tac in patients with IL-2 receptor expressing leukemia and lymphoma, as well as in individuals with graft versus host disease. Bismuth-212, an  $\alpha$ -emitting radionuclide, and Yttrium-90, the  $\beta$ -emitting radionuclide, have been conjugated to anti-Tac using chelates that neither damage the antibody nor permit the elution of radionuclide from it. Following efficacy and toxicity studies in animal models, a dose escalation trial has been initiated with Yttrium-labeled anti-Tac for the treatment of HTLV-I-associated adult T-cell leukemia (ATL). Eleven of the 17 patients underwent a partial or complete remission following Yttrium-90 anti-Tac therapy. One of the limitations of this approach was related to the immunogenicity of the murine antibody. A therapeutic trial has been initiated with  $^{90}\text{Y}$ -humanized anti-Tac.

Dr. Jay Berzofsky continues to study the mechanisms by which T cells recognize antigens presented 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 cancer. He has identified a peptide from the HIV envelope which binds more tightly to a class II MHC molecule, and is effective in mice at 10-100-fold lower doses than the natural sequence in eliciting helper T-cells specific for the natural HIV epitope. He has also found that another HIV peptide is recognized by cytotoxic T lymphocytes (CTL) from 4 strains of mice with 4 different class I MHC molecules. This same HIV peptide is also presented by both class I and class II MHC molecules to CTL and helper T cells, respectively. These epitopes have been utilized to make a synthetic peptide vaccine for HIV. The optimal adjuvant formulation has been determined for a human phase I clinical trial, and toxicology and clinical protocols are in preparation. Dr. Berzofsky's laboratory has also developed a new method of immunizing with peptides without adjuvant, using dendritic cells. They have applied this to cancer vaccine development, and have shown that an endogenously expressed mutant p53 oncoprotein in a tumor cell can serve as a target antigen for CTL, and that such CTL can be elicited by immunization with a synthetic peptide from the mutant p53 sequence. They have also begun determining helper and CTL responses to peptides from human papillomavirus oncoproteins E6 and E7, which should be useful for both diagnosis and possible vaccine treatment of papillomavirus-related cervical cancer. In addition, they have found that the immune defect in asymptomatic HIV-infected patients in their T-cell response in vitro to HIV peptides can be overcome by use of anti-IL-10 antibodies, suggesting a possible approach to therapy.

Dr. Michael Blaese's laboratory continues to focus on the development of gene therapy. He led the group which performed the first authorized use of gene transfer to treat human disease when they infused autologous ADA gene-corrected T cells into two girls with ADA deficiency SCID. More recently, Dr. Blaese and his colleagues have used retroviral-mediated gene-transfer to insert a corrective ADA gene into CD34 selected lymphohematopoietic stem cells. GM-CSF was used to mobilize stem cells into the peripheral blood in a 12-year-old with ADA deficiency. Further, umbilical cord blood was used as a stem cell source in three newborn infants who had been diagnosed with ADA deficiency in utero.

A similar strategy of cellular immunotherapy has now been employed in a clinical trial in AIDS patients. Thirty seven (37) twin pairs discordant for HIV have been enrolled in a study to determine whether normal T-cells could be given to correct the immunodeficiency of AIDS patients and further, whether gene insertion could be used to introduce HIV resistance to these T cells.

Dr. Blaese's laboratory has also developed a unique new approach to direct gene therapy of cancer using inoculation of murine fibroblasts producing retroviral vectors directly into tumors in situ. Using vectors containing the gene for herpes simplex thymidine kinase, he has shown cure of brain tumors in rats following systemic administration of the anti-herpes virus drug, ganciclovir. This antitumor effect was shown to be aided by a "bystander effect" in which phosphorylated ganciclovir is transmitted from tk-gene containing tumor cells to neighboring unmodified tumor cells through "gap junctions" extending delivery of toxin in the treated tumors. A clinical trial is in progress using this approach to treat human glioblastoma or metastatic brain tumors.

**Experimental Immunology Branch**  
**Dr. Alfred Singer, Chief**

The Experimental Immunology Branch (EIB) carries out laboratory investigations in basic immunobiology with particular emphasis on lymphocyte differentiation and regulation; cell biology of immune responses; signal transduction; and the structure, regulation and function of genes involved in immune responses. The EIB flow cytometry laboratory, directed by Susan Sharrow, continues to support multiple investigations which involve quantitative, single cell, multi-parameter immunofluorescence analysis of cells prepared from a variety of tissues and species, as well as a spectrum of in vitro cultured cells.

Dr. Alfred Singer's laboratory has examined the intra-thymic differentiation of functionally and phenotypically distinct T cell subsets, as well as their interaction with thymic epithelium. His studies have provided valuable insight into the development of cytotoxic and helper T cells. The process of negative selection, by which potentially self-reactive T cells are deleted during development, has been analyzed in the laboratory of Dr. Richard Hodes. He has shown that strain-specific deletions in multiple T cell receptor (TCR) V $\beta$  products are related to the expression of multiple MHC and non-MHC self determinants, indicating that maintenance of tolerance to a variety of self determinants results in substantial deletions in the available TCR V $\beta$  repertoire. A previously uncharacterized tumorigenic milk-borne virus in BALB/c mice was found to induce deletion of T cells expressing TCR V $\beta$ 2 in developing mice. This effect was MHC-dependent and suggests that expression of superantigenic capacity is an essential characteristic of infectious MMTV.

Dr Stephen Shaw's laboratory has been systematically analyzing heterogeneity among subsets of human T cells and the functional capacities of those subsets. The concept that adhesion molecules often mark T cell subsets has been confirmed and extended during the past year. The laboratory has continued studies of the T cell integrins which mediate strong adhesion to endothelium. Their studies have strengthened the model that binding occurs via a cascade consisting of at least 3 steps: tether, trigger, and strong adhesion. Dr. Shaw's laboratory has discovered two "pro-adhesive" cytokines which are potential physiologic "triggers": MIP-1 $\beta$  and hepatocyte growth factor (HGF). It has been proposed that these factors are retained at the endothelial surface by binding to proteoglycan. A broad physiologic model has been developed which proposes that such pro-adhesive cytokines are delivered to endothelium by a specialized fibroblastic reticular conduit system.

Dr. Gene Shearer's group has shown both HIV<sup>-</sup> individuals and patients with systemic lupus erythematosus exhibit a spectrum of T helper (TH) functional defects which are predictive for disease progression and are associated with changes in the profiles of immunoregulatory cytokine production, including interleukins 2, 4, 10, and 12, as well as interferon- $\gamma$ . A significant number of HIV-exposed, seronegative individuals from every known risk group were found to exhibit in vitro TH function to synthetic peptides of HIV gp120. Studies in these at-risk groups and newborn infants of HIV<sup>+</sup> mothers suggest that HIV- specific TH function is protective against HIV infection and/or progression to AIDS.



The laboratory of Dr. Dinah Singer continues to characterize the molecular mechanisms regulating MHC class I gene expression. A recently initiated study focuses on the effect of HIV on class I gene expression. It was found that HIV was able to decrease class I promoter activity by up to 12-fold. Repression was mediated specifically by the HIV tat protein which was derived from two coding exons; tat derived from a single coding exon did not repress. These studies define an activity for two-exon tat distinct from that of one-exon tat. They further raise the possibility that during persistent infection, HIV infected cells express reduced levels of class I providing a mechanism whereby they remain hidden from the immune system.

In studies on the mechanism of lymphocyte-mediated cytotoxicity, Dr. Pierre Henkart's laboratory has extended the granule exocytosis model to include a role for granzymes (serine proteases in granules) in triggering "apoptotic" damage to target cells. It was shown that CTL target cells loaded with the protease inhibitor aprotinin were resistant to the lethal hit, while injection of several different proteases into tumor cells triggered a rapid cell death. The data indicate that cells have an internal death pathway responsive to internal proteolysis, and further support the granule exocytosis model for lymphocyte-mediated cytotoxicity. Dr. Henkart's laboratory has also identified a cell death pathway involved in the antigen-induced death of mature T lymphocytes. Their pathway does not operate for steroid-induced programmed cell death of any cells tested, nor for TCR-triggered death of immature thymocytes. Inhibitors, or the calcium-activated cysteine protease, calpain, partially restore defective T helper proliferative responses of cells from HIV donors. These findings have led to the proposal that calpain inhibitors be used for therapy in HIV infection.

Dr. Michael Kuehn's laboratory carries out retroviral insertional mutagenesis to identify genes that have important roles during mouse embryonic development. Mouse embryonic stem (ES) cells are used to import into the mouse germ line large numbers on independent proviral insertions, each of which has the potential to induce an insertional mutation. Previous studies used a single retroviral vector to infect ES cells; insertional mutagenesis studies are now being done using a multiplex approach. He has recently identified a gene that belongs to the TGF $\beta$  superfamily that plays a critical role in the induction and pattern of mesoderm structure in the developing mouse embryo, and may be involved in the induction of tumors.

## **Laboratory of Tumor Immunology and Biology**

### **Dr. Jeffrey Schlom, Chief**

The Laboratory of Tumor Immunology and Biology plans and conducts research on the molecular and immunologic aspects of human cancer. Research programs include the identification and analysis of specific gene products that may potentially be used as targets for cancer immunodiagnosis and immunotherapy; these studies involve the generation and characterization of monoclonal antibodies, and the design, construction and analysis of novel recombinant immunoglobulin molecules.

To date, major emphasis has been placed on the construction of recombinant vaccinia viruses containing human tumor associated genes and in the development of recombinant forms of these gene products in baculovirus. In initial studies a recombinant vaccinia virus containing the human CEA gene has proved to be immunogenic and safe in both rodents and primates, and to elicit good anti-tumor responses in a rodent model. Immunogenicity and safety of this recombinant vaccine has also been tested in non-human primates. The use of a CEA vaccinia virus construct has potential for the active specific immunotherapy of human breast, gastrointestinal and other CEA expressing cancers. Phase I trials have recently been initiated. A recombinant vaccinia virus containing the gene for Prostate Specific Antigen (PSA) is also being developed for use in the immunotherapy of prostatic carcinoma.

Progress has been made in characterizing monoclonal antibodies (MABs) to three carcinoma associated antigens and in determining the potential use of these Mabs in both the diagnosis and therapy of a wide range of carcinomas. The three antigens are (a) TAG-72, a high molecular weight mucin expressed in gastrointestinal, breast, ovarian, endometrial, prostate and non-small cell lung cancers; (b) carcinoembryonic antigen (CEA), a 180,000 D glycoprotein, expressed in gastrointestinal, some breast and some non-small cell lung cancer, and (c) a 48,000 D glycoprotein expressed on colon carcinomas and normal colon, which is recognized by Mab D612. Numerous collaborative Phase I and II clinical trials with MABs B72-3, CC49, COLI, and D612 have been completed or are in progress. A mouse-human chimeric Mab, cD612 Mab, has been developed which has been expressed and secreted by a human T cell line. The secreted immunoglobulin retained its antigen-binding properties and its ability to mediate antibody-dependent cell-mediated cytotoxicity against human tumor cells. To our knowledge, this is the first demonstration of the production of an IgG by human T cells and opens the possibility of a therapeutic approach in which T-cells secrete humanized anti-tumor Mab capable of mediating antibody-dependent cell-mediated cytotoxicity at the tumor site.

The focus in Dr. Barbara Vonderhaar's laboratory has been on the interactions of hormones, antihormones, and growth factors in mammary gland growth and development and tumorigenesis. Recent studies revealed that the nonsteroidal antiestrogens such as tamoxifen (TAM) and nafoxidine, acting through the antilactogen binding site (ALBS), inhibit the binding of prolactin (Prl) to normal mouse mammary membranes. In addition to inhibition of Prl binding, TAM also prevents the Prl-induced accumulation of caseins by cultured mouse mammary explants. These and other data suggest that the ALBS may be one form of the Prl receptor and that TAM and the lactogenic hormones may share a common binding site.

Dr. Robert Callahan's laboratory has focused its efforts on the identification and characterization of frequently occurring mutations in mouse mammary tumor virus (MMTV)-induced mouse mammary tumors as well as in primary human breast tumors. Activation of the Int-3 locus was first detected by this group in the CZECHII mouse mammary tumors. More recently they have identified a new common integration site (designated Int-6) for MMTV in the CZZ-1 mammary hyperplastic outgrowth line. The Int-6 locus is located on chromosome 15 and encodes a 1.4 kb RNA species. Dr. Callahan's group extended its study of primary human breast tumors and they have identified 9 regions of the cellular human genome

that are frequently affected by loss of heterozygosity (LOH). In most cases LOH at a particular locus was associated with tumors bearing more aggressive characteristics.



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In this model the tumors can only be induced in genetically susceptible strains; the inducing agents are not metabolically active; the tumors all have characteristic chromosomal translocations that activate the c-myc proto-oncogene; and the initiated tumor cells require a selective microenvironment (chronic inflammatory tissue in the peritoneum) for progression. Evidence from this system and others indicates that oncogenic mutations can develop and persist long before malignancy. Detection of major oncogenic mutations may provide an experimental system for finding ways to eliminate and control these potentially dangerous cells before they progress to malignancy.

The major new finding this year has been the development by Dr. Siegfried Janz of a sensitive PCR amplification technique for detecting the most common type of translocation T(12;15)-Sa-c-myc. This methodology can detect approximately 60% of the translocations in plasmacytomas. We have used it to study the presence of the translocations in the oil granuloma tissue where PCTs develop and have found that these illegitimate exchanges can be detected as early as 30 days after the injection of pristane. Dr. Janz has also found evidence that chromosomal translocations occurring in the preneoplastic period can be remodelled. We are hoping to refine the sensitivity of the method to be able to determine if specific genes are involved in determining the rate of formation of these translocations. Studies are underway to determine the kinetics of translocation formation and the cell and tissue origin of these translocations.

In a related system, Dr. Linda Wolff continues her study of the mechanisms involved in development of promonocytic leukemia in our murine model induced by a combination of Moloney MuLV and pristane. Using PCR amplification she has shown c-myc activation by insertional mutagenesis occurs early in bone marrow and spleen within the first month following virus inoculation. This year she determined that 1) cells undergoing c-myc activation during the preleukemic period can develop as separate clones at multiple bone marrow sites; 2) some mice that are resistant to disease are unable to support virus replication, as in the case of C57BL/6 mice. Alternately, mice of other resistant strains, for example NFS and C3HeJ, are able to support replication and support c-myc activation, but are blocked in tumor formation at a subsequent unknown step; 3) MuLVs that are nonpathogenic in susceptible strains of mice are capable of integrating at the c-myc locus and forming gag-myc mRNA through a splicing event identical to that utilized by Moloney MuLV; this suggests that, in mice infected with these viruses, the block to leukemia development also occurs at a late stage in the disease process.

Our major focus has been on defining genes that determine susceptibility and resistance to plasmacytoma induction.

Dr. Beverly Mock has analysed inheritance of susceptibility to pristane-induced plasmacytomagenesis in 821 backcross progeny generated between BALB/cAnPt females (Pct-susceptible) and male F1 hybrids (Pct-resistant) between BALB/c and DBA/2. RFLP analyses of a subset of these susceptible backcross progeny for a series of 304 markers/chromosome has indicated linkage of at least one or more of the genes controlling susceptibility to mouse Chr 4. During the past year, RFLP analyses of a series of 23 markers distributed across the entire length of Chr 4 were performed on a series of 77 susceptible and 68 resistant backcross progeny. These analyses position the susceptibility locus/region in the distal part of mouse Chr 4 near the markers D4Lgm3, D4Mit13, Gt-10 and Tnfr-1. We are continuing to isolate random DNA markers from Chr 4 in an effort to generate additional probes for analysis. This

region of mouse Chr 4 is homologous with human Chr 1p and will, therefore, be of interest to examine in multiple myeloma and other cancers involving cytogenetic aberrations in human Chr 1p. Our RFLP analyses have also indicated the possibility of a susceptibility gene in DNA/2 mice (and a resistance gene in BALB/c mice) on Chr 1 (90-95% probability of linkage).

We have found two resistance genes on Chromosome 4. These genes appear to be relatively late acting and inhibit the progression of foci to plasmacytoma.

Dr. Francis Wiener continues his cytogenetic studies on trying to find the B-cell of origin in which the translocations take place. Much of his work takes advantage of a congenic strain, BALB/c.Rb6;15, that develops a high incidence of PCTs following pristane. A substantial portion of these PCTs (unlike EALB/c) carry an inversion (translocation) of chromosomes 6 and 15 involving the Ig kappa and Pvt loci on the Rb6;15 chromosome, indicating the sequestration of the relevant critical chromosome in the nucleus is a factor in facilitating chromosomal translocation.

Dr. Konrad Huppi has identified a 57-bp stretch of DNA, designated Pvt-1a, which comprises part of the largest ORF (140 aa) established to date in Pvt-1 and is consistently found at the amino terminus of alternatively spliced transcripts of mouse Pvt-1. cDNAs obtained by RT-PCR from RNAs of T(6;15) plasmacytomas shows that Pvt-1a is spliced directly, and in frame, to Ig-Ck. Dr. Huppi has identified mutations within the coding region of c-myc in a significant percentage of a large panel of human and mouse tumors. This may be a new mechanism for myc deregulation.

Dr. Emily Shacter has continued her study of the role of IL-6 in the pristane oil granuloma. She has found evidence that indomethacin, a powerful inhibitor of plasmacytomagenesis in BALB/c mice also strikingly but not completely inhibits IL-6 secretion by peritoneal macrophages. The stimulator of IL-6 production *in vivo* has not yet been identified. Dr. Shacter, however, has found that albumin polymers can produce dramatic increases in IL-6 production.

Dr. Stuart Rudikoff has extended the study of PCT formation by the myc-refractory J3V1 retrovirus. BALB/c mice are highly susceptible to plasmacytoma (PCT) induction protocols using the myc-raf containing J3V1 retrovirus while DBA/2 mice are resistant. We have previously demonstrated that susceptibility is associated with the BALB/c B cell genotype. To examine the role of T cells in the pristane microenvironment, J3V1 was used to induce tumors in nude mice. B lineage tumors in nude mice were almost exclusively pre-B or B cell by phenotype with PCTs occurring only rarely. In contrast, when tumors were induced in T cell reconstituted nude mice, 40% were PCTs and 60% were B cell tumors indistinguishable from those induced in unreconstituted nude mice. These results are interpreted to indicate that J3V1 can transform early B lineage cells and that T cells play no role in the transformation process. T cells are, however, required to drive transformed B cells into fully differentiated, antibody secreting plasma cells.

We have recently found a new potent plasmacytomagenic agent, dimethylpolysiloxane, the principal component of the gels used in breast implants. This material in relatively small amounts induces the formation of a granulomatous tissue on mesenteric surfaces.

Dr. Wendy Davidson has established and extended a study of the mice homozygous for *lpr* (a defect at the *fas* locus) and *gld* that develop autoimmunity and a profound lymphadenopathy. This lymphoproliferative disease is characterized by the accumulation of two functionally anergic T cell subsets, a predominant B220<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> (DN) population and a minor B220<sup>+</sup>CD4<sup>+</sup> population. This system is a model for understanding how anergic lymphocytes can accumulate *in vivo*. Depletion of CD8<sup>+</sup> T cells *in vivo* and ongoing adoptive transfer of *gld* lymphocyte subsets into *scid* mice revealed that: 1)

CD8<sup>+</sup> T cells are required for the accumulation of DN T cells; 2) the precursors of DN T cells are present in gld BM, LN and spleen; 3) CD4<sup>+</sup> and CD4<sup>+</sup>B220<sup>+</sup> T cells do not spontaneously differentiate into DN T cells; and 4) DN T cells do not grow autonomously in scid mice or differentiate into CD4<sup>+</sup> or CD8<sup>+</sup> T cells. She has found evidence that DN T cells may be able to proliferate in vivo under special conditions. On the basis of these and earlier studies, she proposes that DN T cells arise from peripheral, previously activated CD4<sup>+</sup>, CD8<sup>+</sup> or CD4<sup>+</sup> CD8<sup>+</sup> T cells that have downregulated the expression of CD4, CD8 and the TCR and normally would be deleted by a fas-dependent mechanism.

Dr. Sandra Smith-Gill has solved and refined the X-ray structures of HyHEL-5 Fab complexed with 2 single site-directed mutants of HEL. One of these reduces the affinity of the complex by over a thousand fold and the other 10 fold. These results represent the first time structures have been obtained for 3 antigens, differing at only a single critical residue, complexed to the same antibody, and will provide valuable insight about the role of Arginine side chains in protein-protein interactions. Experiments with specific-pathogen-free and conventional BALB/c mice have established that plasmacytoma-refractory SPF BALB/cAnPt mice have naive T cell responses as well as associated restricted B cell responses. These results suggest a significant influence of antigenic exposure, particularly viral antigens, on development of the specificity repertoire and plasmacytomagenesis.

Dr. J.F. Mushinski, along with Dr. Harald Mischak, has isolated all of the PKC isoforms in the mouse and studied their expression in a series of B-cell tumors. Overexpression of PKC- $\delta$  appears to slow the growth of NIH3T3 cells; only PKC- $\alpha$  and PKC- $\delta$  induced the 32C cell line and 32D to differentiate.

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

PROJECT NUMBER

Z01 CB 05553-24 LG

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Plasmacytomagenesis and lymphocyte development

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

P. I.:	S. Rudikoff	Microbiologist	LG, NCI
	P. Hausner	Visiting Associate	LG, NCI
	D. Hilbert	Staff Fellow	LG, NCI
	V. Roschke	Visiting Associate	LG, NCI
	M. Shen	Visiting Fellow	LG, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

Molecular Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

5

PROFESSIONAL:

3

OTHER:

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.)

I. BALB/c mice are highly susceptible to plasmacytoma (PCT) induction protocols using the myc-rf containing J3V1 retrovirus while DBA/2 mice are resistant. We have previously demonstrated that susceptibility is associated with the BALB/c B cell genotype. However, tumor induction still requires granuloma formation initiated by the prior administration of pristane indicating the necessity for an appropriate microenvironment for neoplastic progression. To examine the role of T cells in this microenvironment, J3V1 was used to induce tumors in nude mice. B lineage tumors in nude mice were almost exclusively pre-B or B cell by phenotype with PCT's occurring only rarely. In contrast, when tumors were induced in T cell reconstituted nude mice, 40% were PCT's and 60% were B cell tumors indistinguishable from those induced in unreconstituted nude mice. These results are interpreted to indicate that J3V1 can transform early B lineage cells and that T cells play no role in the transformation process. T cells are, however, required to drive transformed B cells into fully differentiated, antibody secreting plasma cells. II. The early development of PCT's is currently being addressed by in situ hybridization studies to assess clonality of developing neoplastic lesions. Probes have been prepared to 7 VH families and have been hybridized to serial sections from granulomatous tissue containing putative early stage lesions. Similar studies will additionally enumerate other cell types associated with developing lesions to define the cellular microenvironment associated with this neoplastic process. III. Previous studies have demonstrated that primary plasmacytomas are dependent on physical contact with stromal cell feeder layers for survival and proliferation. This interaction is mediated, in part, by the cell surface molecule CD44, but available antibodies to other mouse cell surface receptors fail to inhibit adhesion. To identify additional molecules involved in this adhesion, rat monoclonal antibodies have been prepared against stromal cells obtained from the primary site of plasmacytomagenesis. These antibodies are currently being screened for specificity and ability to inhibit adhesion of plasma cells to stromal cell feeder layers. The role of CD44 in both neoplastic and normal development is a question of major biological interest. To examine the *in vivo* function of CD44 we are in the process of generating CD44 knockout mice. A series of genomic clones have been isolated and are currently being analyzed for exon locations. A selected exon will then be interrupted with the neomycin resistance gene and the inactivated allele introduced into embryonal stem cells for generation of chimeric mice.



Major Findings:

I. The prototypic susceptible and resistant mouse strains for plasmacytoma induction are BALB/c and DBA/2, respectively. In the Annual Report '92 we described studies addressing questions of the cellular basis of plasmacytoma development by cell transfer experiments into SCID mice. Results demonstrated that the susceptible phenotype for J3V1 retroviral induced plasmacytomagenesis was directly associated with the BALB/c B cell genotype. However, it is clear that the microenvironment also plays a critical role in plasmacytoma development as J3V1 successfully induces tumors only in pristane primed animals. The nature of the signals provided by the pristane induced granuloma is unclear, but numerous cell types including B cells, T cells, macrophages, neutrophils, and stromal elements are found in this tissue. In an effort to further define cellular contributions, particularly that of T cells, to plasmacytoma development, tumors have been induced in BALB/c nude mice lacking functional T cells and compared to those arising in immunocompetent animals.

In initial induction experiments, the tumor incidence was found to be 53% in normal BALB/c animals and 96% in nudes although the proportion of B lineage tumors in nudes was only 38% compared to 75% in BALB/c. The non-B lineage tumors are almost exclusively myeloid. Histologic, molecular, and cell surface analysis revealed that the BALB/c tumors were classic plasmacytomas. In contrast, the B lineage tumors arising in nude mice were predominantly large cell lymphomas that formed nodules of densely packed cells within the granuloma in a completely different manner than plasmacytomas. The nude tumors largely fail to express the ThB antigen found on nearly all plasmacytomas, do not secrete immunoglobulin and frequently have immunoglobulin genes in the germline configuration. These data indicate that the predominant type of nude tumor is that of either a pre-B or B cell that fails to differentiate into a mature plasma cell. To formally distinguish between the possibility that T cells play a role in plasmacytoma development or that the above described tumors were a result of the nude mutation, a second series of inductions was performed using nude mice reconstituted with purified lymph node T cells. Analysis of 15 B lineage tumors arising in T cell reconstituted mice revealed that 40% are plasmacytomas as defined by expression of the ThB antigen and immunoglobulin secretion while the remaining 60% are phenotypically identical to the pre-B, B cell tumors previously observed. It thus appears that T cells are not required for the transformation process, but function to drive transformed B cells into terminally differentiated plasma cells. We are currently attempting to increase the number of B lineage tumors in this system by use of retroviral stocks lacking the MuLV helper virus and infection of purified B cells *in vitro*.

To further examine the characteristics and growth requirements of the nude B lineage tumors, cell lines have been established and compared to those obtained from BALB/c mice. BALB/c lines are slow growing, initially require the presence of stromal feeder layers, and are IL-6 dependent. In contrast, the B lineage nude tumors grow rapidly, do not require stromal cells, and are IL-6 independent. Some of these lines appear to rearrange immunoglobulin genes *in vitro*. Interestingly, both the plasmacytomas and B lineage tumors can only be passaged *in vivo* in pristane primed recipients. We are currently exploring gene expression in these two tumor types using a differential display technique to identify transcripts unique to a particular phenotype.

II. While plasma cell tumors have been extensively studied and characterized, correspondingly little is known about the early *in vivo* progression of these neoplasias. To examine this aspect of tumorigenesis, we have developed multi-color *in situ* hybridization techniques to assess early stages of plasmacytomagenesis. A series of paraffin sections have been prepared from mice at various times post pristane injection, some of which have been characterized by Dr. Michael Potter for the presence of plasma cell 'foci' which can be identified in the susceptible BALB/c strain. In the first series of experiments, these samples have been hybridized with kappa and lambda constant region probes

to enumerate all plasma cells and then a series of heavy chain variable region probes to determine clonality of developing lymphoid aggregates and plasma cell foci. These experiments are nearly complete and data analysis is currently underway. Serial sections will subsequently be hybridized with a number of additional probes to identify other cell types associated with plasma cells and the growth factors - receptors they express. Preliminary experiments using plasmacytoma cell lines indicate that chromosomal translocations can be readily and selectively detected due to transcription from both the sense and non-sense strands. Serial sections will, again, be hybridized with these probes in an attempt to identify the presence of translocations in developing plasma cells.

III. We have previously demonstrated that primary plasmacytomas are completely dependent on stromal cells from the site of tumor development for *in vitro* survival and growth. The stromal cells supply at least two signals required by the plasma cells, one being IL-6, and the second, of unknown nature, requiring direct contact between the two cell types. The adhesion of plasma to stromal cells is, in part, mediated by CD44 as antibodies to CD44 partially inhibit adhesion of plasma cells to stromal layers. To further characterize the interaction between plasma and stromal cells, we are currently preparing and analyzing monoclonal antibodies obtained by immunization with previously characterized stromal cell lines. Rats have been immunized with the 5-25 stromal cell line and hybridomas prepared by fusion with appropriate rat or mouse lines. To date, 107 rat x rat and 139 rat x mouse hybrids have been obtained. Since the stromal cells are tightly adherent, FACScan and ELISA analysis are of limited value due to the alteration of cell surface molecules during detachment and assay. We have, therefore, developed a direct cell binding assay which is used in conjunction with FACScan and ELISA. Preliminary analysis of the rat x rat hybrids reveals that of 107, 67 are positive on stromal cells and 20 of these are negative with plasma cell lines. Of these, one is also negative on a bone marrow stromal cell line suggesting possible specificity differences among stromal cells from different sources. Production and analysis of stromal cell specific hybridomas is continuing with the intent of defining adhesion molecules on these cell surfaces involved in lymphocyte attachment and growth stimulation.

IV. Our previous finding that CD44 was partially responsible for the adhesion of plasma cells to stromal layers as well as its described role in metastasis has generated an interest in examining the *in vivo* function of this molecule. To this end we have begun studies aimed at generating a CD44 'knockout' mouse. A CD44 probe has been generated by reverse transcriptase PCR and used to screen a genomic library. Six positive clones have been obtained and exon specific probes are being used for characterization. Once mapping is complete, one of the clones will be selected for insertion of the neomycin resistance gene into the appropriate exon and, in collaboration with Dr. Michael Keuhn, LEI, NCI the inactivated gene will be introduced into embryonic stem cells for the generation of 'knockout' mice. We are also simultaneously attempting to perform similar experiments with the IgA switch region to generate animals incapable of expressing IgA immunoglobulins. These mice will be used as a model for IgA deficiency as well as to examine plasmacytoma generation in the absence of IgA, the most frequent class of immunoglobulin expressed in this disease.

#### Publications:

Degrassi A, Hilbert DM, Rudikoff S, Anderson AO, Potter M, Coon HG. *In vitro* culture of primary plasmacytomas requires stromal cell feeder layers. *Proc Natl Acad Sci USA* 1993;90:2060-4.

Henderson TJ, Rudikoff S. Characterization of a VK subfamily in Mus musculus castaneus: expansion at the subset level. *Immunogenetics* 1993;37:415-25.

Henderson TJ, Rudikoff S. Characterization of a VK family in Mus musculus castaneus: sequence analysis. *Immunogenetics* 1993;37:426-36.

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Hilbert DM, Pumphrey JG, Troppmair J, Rapp UR, Rudikoff S. Susceptibility and resistance to J3V1 retrovirus induced murine plasmacytomagenesis in reconstituted SCID mice. *Oncogene* 1993;in press.

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.)

**Pathogenesis of plasma cell neoplasia: resistance and susceptibility genes**

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

P.I.:	M. Potter	Chief, Lab. of Genetics	LG, NCI
	E.B. Mushinski	Bio. Lab. Tech.	LG, NCI
	G. Jones	Bio. Lab. Tech.	LG, NCI
	B. Mock	Staff Fellow	LG, NCI
	E. Shacter	Expert	LG, NCI
	S. Janz	Visiting Associate	LG, NCI
	F. Wiener	Visiting Scientist	LG, NCI
	H. Hennings	Biologist	DCE, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS:

15

PROFESSIONAL:

6

OTHER:

9

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 major project in our laboratory is to determine pathogenetic mechanisms involved in the induction of plasmacytomas by the intraperitoneal introduction of various substances (e.g., paraffin oils, solid plastic discs). We have recently found a new potent plasmacytomagenic agent dimethylpolysiloxane, the principle component of the gels used in breast implants. This material in relatively small amounts induces the formation of a granulomatous tissue on mesenteric surfaces. Our major focus has been on defining genes that determine susceptibility and resistance to plasmacytoma induction. We have found two resistance genes on Chromosome 4. These genes appear to be relatively late acting and inhibit the progression of foci to plasmacytoma. We continue to refine our focus assay system for the earlier detection of susceptibility and resistance. This work has been delayed because of the apparent development of a mutation in our BALE/canPt colony that partially confers resistance to plasmacytoma induction. In collaboration with Siegfried Janz a PCR amplification system has been developed for the detecting of IgSa-C-myc illegitimate recombinations that occur in the chromosomal translocation T(12;15) which is found in 90% of mouse plasmacytomas induced by paraffin oils. We are currently evaluating whether the frequency of these translocations is favored in plasmacytoma susceptible strains.

## I. Pathogenesis of Plasma Cell Neoplasia - Dr. Michael Potter

Environmental and Genetic Factors: In 1988-89 we initiated our production colony of BALB/cAnPt mice from a single pair in accordance with accepted procedures; this subline is designated "A". The mice in this subdivision of the colony have exhibited a reduction in plasmacytoma incidence from 60% to 30% at 300 days. At first this was thought to be due to the increased isolation and cleanliness of the colony, but evidence obtained in the last year has shown that other sublines of BALB/cAnPt"B", as well as certain C.D2 plasmacytoma-susceptible congenics which have been used in coincidental studies, have given the expected high incidence of plasma cell tumors. While the SPF state is associated with an extreme reduction in the incidence of plasmacytomas, i.e. to 5%, this recent data indicates that a mutational change resulting from genetic drift has taken place in our colony, and that affects plasmacytoma incidence. We have not been able to confirm the effect of immunization on plasmacytoma formation and have temporarily discontinued these experiments.

We have continued to study the pathology of plasmacytoma formation by studying inducing agents other than paraffin oils. Rough-edged plastic discs approximately 2 cm in diameter induce plasmacytomas. Unfortunately, our experiment was set up in BALB/cAnPt"A" mice primarily to study the relationship of the peritoneal reactive tissue to plasmacytoma formation, and we did not obtain an incidence. These tumors appeared to arise in the omentum and are associated with the milk spots (lymphoid aggregates), and there was a less clear association with the reactive fibrous tissue. The paucity of reactive tissue presented a striking contrast to the oil granuloma. We have now identified a new plasmacytomagenic agent, the silicone gel used in breast implants. The intraperitoneal injection of 0.4 ml of this very sticky material results in the formation of a 'glob' of gel that floats freely in the peritoneum. It appears to be only mildly inflammatory and is well tolerated by the mice. After 4-6 months the glob can be lifted out as a single mass; however, microscopic studies have shown that small fragments of the silicone gel have broken off and seeded onto mesenteric surfaces where they induce the formation of a granulomatous tissue that resembles the oil granuloma. As with the discs, relatively little granuloma tissue is formed. The BALB/cAnPt"A" mice have, however, developed plasmacytomas which are associated with this granuloma tissue, and the incidence thus far is about 20% at 300 days. We expect this to increase when data on more susceptible sublines become available. We are carrying out studies in collaboration with Dr. Fred Miller, Division of Biologics Standards, on the pathological effects of silicone gels. In addition we will now begin studying the liquid forms of poly di-methyl siloxane that vary in viscosity from 100 to 60,000 centistokes.

Plasmacytoma induction experiments require usually 300 days to establish an incidence; however, focal proliferations of atypical plasma cells can be detected in tissue sections of oil granuloma tissue beginning around 60 days, and by day 50 the percentage of BALB/cAn mice with multiple foci is high enough that the number of foci per mouse can be used to determine susceptibility or resistance to plasmacytoma development. We are now completing this long quantitative study of foci that shows that we can use the 150 day focus assay. This assay is particularly useful in genetic studies.

Genetic Studies: We are continuing to develop and characterize C.D2 congenic mice to search for genes that determine resistance to plasmacytomagenesis.

The chromosome 4 (Chr4) congenics have been studied in detail to obtain different segments of this chromosome. We have strong data now that indicate there are two discontinuous PCT resistance genes on the distal half of Chr4. The presence of only one of these PCT-R genes can produce a significant resistance to plasmacytoma induction. This suggests that susceptibility requires the cooperation of at least 2 (PCT-S) genes. We will refine the

regions where these genes are located to the point where the minimum amount of DBA/2 chromatin is 5 centimorgans or less. This work is being done in collaboration with Beverly Mock who has extensively mapped allelomorph differences between BALB/c and DBA/2 on Chr4.

We are now in the process of screening the non-chromosome 4 congenics for susceptibility and resistance.

Cellular Origin of PCTs: In collaboration with Jim Kenny we are carrying out induction experiments on BALB/c.CBA-xid/xid mice. This congenic strain was developed by Karl Hansen, and our mice are derived from a late backcross (N18-20). These mice have developed a very low incidence of PCTs, i.e. <5%. XID mice have a marked reduction in B cells but also specifically lack the peritoneal Ly-1B cells. This provides some evidence that the normal long lived and self-renewing peritoneal Ly1 B cells may be the precursors of plasmacytomas. We are pursuing this further in collaborative experiments with Francis Weiner in which BALB/cAn.CBA-xid/xid mice will be given cytogenetically normal BALB/c cells to determine if both the peritoneal B population as well as plasmacytoma susceptibility can be restored.

Genetics of Chromosomal Translocations: Siegfried Janz has developed a PCR amplification technique for detecting the most common type of translocation T(12;15)-Sa-c-myc. This methodology can detect approximately 60% of the translocations in plasmacytomas. We have used it to study the presence of the translocations in the oil granuloma tissue where PCTs develop and have found that these illegitimate exchanges can be detected as early as 30 days after the injection of pristane. We are hoping to refine the sensitivity of the method to be able to determine if specific genes are involved in determining the rate of formation of these translocations. Studies are underway to determine the kinetics of translocation formation, and the cell and tissue origin of these translocations. We have discontinued working on the preferential repair assay system of Vilhelm Bohr as genetic assay. Gary Jones is now able to carry out the PCR assay system and is working intensively on evaluating the incidence of translocations in the oil granuloma tissues of the BALB/c sublines, C.D2-DBA/2-Chr4 congenics and the hypersusceptible C.D2-DBA/2 congenic strains.

Genetics of Skin Carcinogenesis: In collaboration with Henry Hennings and Stuart Yuspa in DCE we have developed inbred SENCAR strains A,B, and C. These mice have proven to develop a high incidence of papillomas and carcinomas using a basic regimen of a single application of 2 ug DMBA and 2 ug TPA given 3X/week for 7 weeks. In contrast BALB/cAnPt mice develop a negligible number of these tumors. Currently we are studying the F1 hybrids of BALB/c X SENCAR A which have proven to be resistant like BALB/c and the first generation backcross to SENCAR to determine how many resistance genes are involved.

Chromosome 11 Studies: Rose-Marie de Kruff at Stanford identified a striking difference in the production of IL-4 by the TH cells of BALB/c and DBA/2 and accordingly we sent her several of our C.D2-DBA/2 congenics to determine if they carried the DBA/2 gene. The C.D2-Es-Hba was found to have the DBA/2 phenotype and we are currently developing a series of recombinants to localize this gene. Beverly Mock has evidence that C.D2-Es-Hba carries a PCT R gene and also a gene that may confer resistance to L-majox.

## II. Cytogenetic Studies on Mouse Plasmacytomagenesis - Dr. Francis Wiener

Plasmacytoma induction experiments in BALB/cRb6.15 mice: Plasmacytomas (PCTs) that arose in pristane + A-MuLV treated homozygous BALB/cRb6.15 mice (hereafter B/c.6.15) carry a high number of variant T(6;15) translocations in the form of invRb(6.15). Similarly, PCTs induced in (B/c.6.15 x BALB/c)F1 mice display an analogous of variant PCTs was due either to the involvement of

the Abelson virus in the translocation process or to the topological relationship between the MYC and Ig-kappa genes in the B/c.6.15 plasmacytoma precursor cells.

To decide between one or the other of these alternatives, PCTs were induced in homozygous B/c.6.15 and BALB/c mice by pristane alone (70 mice per group). The PC incidence in B/c.6.15 and BALB/c groups at day 267 was 77.4% and 44.2%, respectively. To date 30 B/c.6.15 and 17 BALB/c PCTs were karyotyped. The main results are as follows:

- 1) The ratio typical versus variant translocation in the B/c.6.15 group is 13:9 while in the BALB/c group it is 17:1.
- 2) If we consider arbitrary two latency stages, days 0 to 172 and days 172-267, the ratio in the B/c.6.15 group is 11:4 and 3:9, respectively. Thus, in the first stage the number of PCTs carrying variant inRb(6.15) translocation is nearly 3 times as high as that of typical ones.
- 3) There are 3 translocation-free PCTs among 30 PCTs of the B/c.6.15 group (10%). This high frequency in only pristane-treated mice is unexpected.
- 4) The ratio of typical vs variant translocation in the BALB/c6.15 group is roughly 1:1, similar to that recorded in the pristane + AM-MuLV induced PCTs.

The BALB/c6.15 mouse differs from the conventional BALB/c by two parameters: 1) a number of AKR derived genes were retained on Chr15 and Chr6, and 2) the distance between the MYC and Kappa gene is constant. Both genes are located on the same Rb6.15 chromosome.

The eventual role of the AKR genes in the proneness of BALB/c6.15 to produce variant inRb(6;15) translocation is difficult to prove but could not be excluded.

It is more likely that the high incidence of the variant type translocation in the B/c6.15 mice is due to the topological relationship between the MYC and Kappa genes in the PCT precursor cells. In the BALB/c cells the distance between these two genes involved in the variant translocation is random because they are carried on disparate chromosomes located in different sectors of the nuclear matrix. In the B/c.6.15 the distance is always constant, i.e., non-random. Both genes are positioned on a single chromosome, namely on the long and short arm of the centromerically fused 6.15 chromosome. Evidently, the chance for illegitimate recombination is higher when the genes are on the same chromosome than on different ones.

The reason for the early occurrence of the variant translocation vs the late appearance of typical translocation in the B/c.6.15 mouse is not clearly understood yet. One may assume that in the early stages following pristane treatment the PCTs develop from precursors being in proB/preB stage of B-cell development residing primarily in the bone marrow. In these cells the rearrangement of the light chains is in progress, consequently the MYC and Kappa genes are more accessible for illegitimate recombination. In later stages the PCTs evolve from maturing B-cells that accumulate in the preneoplastic foci of the peritoneal cavity. In these cells the illegitimate recombination is probably associated with the IgH switching process.

The significance of trisomy 11 in PC development: Cytogenetic analysis of PCT induced in BALB/c and congenic mouse strains revealed that trisomy 11 was the second most frequent chromosomal change besides the PCT-associated translocation. Interestingly, its frequency was relatively higher in PCTs induced by pristane + A-MuLV compared to that induced by pristane alone. Its biological role in mouse plasmacytomagenesis is not yet clearly understood.

The question that we asked was whether the high frequency of trisomy 11 1) is restricted to Abelson virus induced PCTs in which the MYC gene is

constitutively activated by chromosomal translocation, or 2) occurs independently of the translocation process. Cytogenetic analysis of translocation free plasmacytomas with constitutively activated exogenous MYC or MYC + ABL may indicate which of the alternatives is more likely to occur.

PCT induction was performed in the following experimental system: SCID mice were reconstituted with bone marrow and/or spleen cells of (BALB/cRb6.15 x DBA/2N)F1 origin. This F1 combination is resistant to pristane induced PCT-gensis. Four weeks after reconstitution PCTs were induced by pristane followed by ABL-MYC virus infection (the ABL-MYC, an A-MuLV based retrovirus that constitutively express both v-abl and c-myc). PCTs induced by J3 virus in BALB/c mice (from an earlier experiment) served as controls. The J3 viral construct contains a hybrid avian v-myc and a hybrid avian and mouse v-raf oncogene.

Overall, the results of the cytogenetic analyses confirm that trisomy 11 was the most consistent chromosomal aberration in ABPCs and ABMYC PCTs in which both myc and abl genes are constitutively expressed. Pristane-only and J3 PCTs with deregulated endogenous or exogenous MYC expression lacked trisomy 11. The results raise the question of whether the accelerated development of the ABPC tumors is determined by a dosage effect due to duplication of a gene(s) located on Chr11. In this context the likely candidates are the cluster of genes on Chr11 that determine the production of a variety of growth factors involved in B-cell development. Cytogenetic mapping of the duplicating segment of Chr11 will allow us to approximate the gene(s) supposedly involved in the acceleration process.

Further studies on tissue origin and characterization of the PCT precursor cell: The bone marrow of the SCID mice contain stem cells that could generate the whole erythroid and myeloid lineage and preB- and preT-cells as well. Irrespective of age, the SCID mice generate a number of competent B- and T-cells designated as "leaky" cells. The aim of the experiment is to answer two questions: 1) whether the SCID-derived proB/preB-cells in which the assembling of functional Ig- $\mu$  heavy chain is prevented by an apparent VDJ recombination defect could develop into non-Ig producing "sterile" but malignant PCTs, and 2) whether "leaky" B cells which possess productively rearranged IgH and IgL chains could serve as PCT precursors.

The experimental design was as follows: (BALB/cRb6.15 x DBA/N2)F1 mice were sublethally irradiated (900 r) and reconstituted with spleen and bone marrow cells from SCID mice. This F1 combination is resistant to PCT genesis but supports PCT development. A control group of sublethally irradiated BALB/cRb6.15 mice was reconstituted similar to the experimental group. The chimeric status of the mice was ascertained by chromosomal typing of the peritoneal cavity cells, bone marrow and spleen based on the presence or absence of an Rb6.15 chromosome. PCTs were induced by pristane alone or pristane + A-MuLV.

The preliminary results are as follows:

- 1) Chromosomal typing performed at different time intervals up to 8 months clearly showed the presence of SCID derived cells in the peritoneal cavity, spleen and bone marrow of the reconstituted mice.
- 2) In the F1 combination two types of SCID cells-derived tumors evolved: i) monocytic tumors that developed in the peritoneal cavity of the chimeric mice, and ii) early T cell type tumors that arose in the spleen of the reconstituted F1 mice. These tumors histopathologically were similar to spontaneous thymomas arising in the thymus of aged SCID mice.
- 3) In the control group PCTs did develop but all were of BALB/cRb6.15 (host) derived. Interestingly, these PCTs arose with short latency at 21 days after viral infection. One of the PCTs has an exceptional



chromosome constitution carrying both the typical T(12;15) and the variant inRb(6;15) translocation.

The conclusion drawn from this experiment is that none of the cells belonging to the B-cell compartment of the SCID mouse contain PCT precursor cells. Alternatively, illegitimate recombination may occur in SCID proB/preB cells, too. However, these cells could not progress into a full-fledged plasmacytoma cell. Seemingly, the development of the malignant phenotype and B-cell differentiation are linked in plasmacytomagenesis.

### III. The Genetic Control of Plasmacytomagenesis - Dr. Beverly Mock

The inheritance of susceptibility to pristane-induced plasmacytomagenesis has been examined in 821 backcross progeny generated between BALB/cANPt females (Pct-susceptible) and male F1 hybrids (Pct-resistant) between BALB/c and DBA/2. RFLP analyses of a subset of these susceptible backcross progeny for a series of 3-4 markers/chromosome has indicated linkage of at least one or more of the genes controlling susceptibility to mouse Chromosome 4. During the past year, RFLP analyses of a series of 23 markers distributed across the entire length of Chromosome 4 were performed on a series of 77 susceptible and 68 resistant backcross progeny. These analyses position the susceptibility locus/region in the distal part of mouse Chr 4 near the markers D4Lgm3, D4Mit13, Gt-10 and Tnfr-1. We are continuing to isolate random DNA markers from Chr 4 in an effort to generate additional probes for analysis. This region of mouse Chr 4 is homologous with human Chr 1p and will, therefore, be of interest to examine in multiple myeloma and other cancers involving cytogenetic aberrations in human Chr 1p. Our RFLP analyses have also indicated the possibility of a susceptibility gene in DBA/2 mice (and a resistance gene in BALB/c mice) on Chr 1 (90-95% probability of linkage).

An alternate method of plasmacytoma induction has involved the inoculation of retroviral vectors carrying differing combinations of oncogenes in addition to small and single doses of pristane. Tumors usually arise within 30-60 days post-inoculation under this induction protocol; the normal latency period for pristane-induced tumors averages between 220-260 days. Previous experiments with the RIM retroviral vector, which carries ras and myc sequences, the AM vector, which carries abl and myc sequences, and the J3V1 vector, which carries raf and myc sequences, have shown that BALB/c mice are susceptible and DBA/2 mice are resistant to tumor induction under these protocols; in addition, the genetic control appears to be modulated by a single gene in the RIM system. Backcross experiments have not been performed with the AM or J3V1 vectors. In order to determine the chromosomal location of the susceptibility/resistance gene in the RIM system, and to determine if the same gene may influence tumor development in the AM and J3V1 systems, a series of bilineal C.D2 congenic strains of mice, harboring DBA/2 donor genes from a variety of different chromosomes, have been and are being evaluated for their S/R phenotypes following inoculation with pristane plus each of these retroviral vectors. Preliminary studies involving at least one experiment/strain tested have indicated the possibility that a region of Chr 17 may harbor a resistance gene in mice which have been infected with either the RIM or J3V1 retroviral vectors. This region has not been implicated in strains of mice receiving the AM retroviral vectors. We have not directly identified a chromosomal region imparting resistance to the AM-induced tumors; however, a mouse strain harboring a region of Chr 11 from the DBA/2 donor strain on a BALB/c background exhibits the possibility of an accelerating effect in the development of tumors. The mean latency period of tumor development in the Chr 11 congenic is significantly shorter than that seen in the BALB/c congener.

IV. Potential Neoplastic Consequences of Chronic Inflammation: Mouse Plasmacytomagenesis as a Model - Dr. Emily Shacter

Summary of research:

Experiments are in progress to determine how chronic inflammation contributes to the development of B cell tumors in pristane-treated mice. Two areas of investigation are being pursued:

(1) Studies on interleukin 6 (IL-6). IL-6 is an inflammatory cytokine that is an essential growth factor for mouse plasmacytoma cells *in vitro*. In previous experiments, we found that chronic high levels of intraperitoneal IL-6 are generated in pristane-treated mice. This is due to continuous production of the cytokine by pristane-elicited macrophages. The goal of the present studies is to identify the endogenous factors responsible for stimulating abnormal IL-6 production in the mice. Knowledge obtained from these studies is expected to give insight into the development of pathological IL-6 levels in human diseases such as multiple myeloma and rheumatoid arthritis. Three experimental approaches are being followed:

(a) Determine how the non-steroidal anti-inflammatory drug indomethacin inhibits pristane-induced plasmacytomagenesis. The mechanism for this inhibition is being sought as a means to identify the pathways that are required for tumor development. In previous studies, we found that indomethacin dramatically diminishes the levels of IL-6 achieved in pristane-treated mice and inhibits IL-6 secretion by peritoneal macrophages. Because the main biochemical function of indomethacin is the inhibition of cyclooxygenase activity, this result suggests that prostaglandins may be involved in mediating the abnormal elevation of IL-6 seen in pristane-treated mice. Experiments are underway to investigate the possible co-regulation of IL-6 and PGE<sub>2</sub> production by peritoneal macrophages.

(b) Quantify the extent to which different inbred strains of mice generate intraperitoneal IL-6 in response to pristane. Significant heritable differences have been found. In particular, plasmacytoma-susceptible BALB/c mice generate the highest levels of IL-6 in response to pristane whereas some plasmacytoma resistant strains are low or non-responders. In addition to providing clues that may help explain the unique genetic susceptibility of BALB/c mice to development of plasma cell tumors, the strain comparisons provide a means to identify factors that are responsible for inducing the high IL-6 *in vivo*.

(c) Investigate whether modified proteins generated as a consequence of the inflammation induced by pristane can stimulate macrophages to secrete IL-6. Indirect evidence for this possibility comes from our finding that albumin polymers stimulate macrophage IL-6 and PGE<sub>2</sub> secretion *in vitro*. This constitutes a novel mechanism for regulation of IL-6 and raises the possibility that albumin polymers or some other physiological counterpart might regulate IL-6 production under some conditions *in vivo*.

(2) Studies on oxidative stress. The prolonged inflammation induced by pristane is characterized by chronic neutrophilia. Previous studies indicated that these cells have the capacity to induce extensive DNA damage in neighboring B lymphocytes when activated to undergo an oxidative burst *in vitro*. However, the extent to which pristane-elicited neutrophils are activated *in vivo* needs to be assessed before a role of neutrophil-derived oxidants in tumor development can be established. Experiments have been carried out to measure protein oxidation in pristane-treated mice as a marker for neutrophil activity. In collaboration with Dr. R. Levine (Laboratory of Biochemistry, NHLBI), we found that DNPH-reactive carbonyl groups accumulate in the peritoneal cavities of pristane-treated mice and that these moieties segregate from the bulk of the exudate proteins in gel filtration HPLC. To

further identify these compounds, a novel Western blot immunoassay for oxidized proteins was developed. The method, which detects each individual oxidized protein in a biological sample, will be useful for the study of biomedical conditions in which protein oxidation plays a role. Application of this method to the pristane-induced inflammatory exudate revealed that the carbonyl compounds induced by pristane may not be protein-associated. Additional experiments are required to identify the carbonyl groups induced by pristane.

V. DNA Damage and Activation of c-myc in Murine Plasmacytomagenesis -  
Dr. Siegfried Janz

Oxidative DNA damage and mechanisms of action of pristane. I have been collaborating with Dr. Emily Shacter on the potential role of DNA damage and repair in plasmacytomagenesis in BALB/c mice. The studies were primarily focused on neutrophil-mediated DNA damage in B lymphocytes. For the first time we provided direct experimental evidence that activated neutrophils undergoing an oxidative burst induce unscheduled DNA synthesis in murine B cells (Janz and Shacter, *Mutat. Res.* 293:173-186, 1993). In order to study more basic aspects of oxidative DNA damage that are relevant for explaining mechanisms of phagocyte-mediated damage, a short-term bacterial genotoxicity assay (Muller and Janz, *Environ. Mol. Mutagen.* 20:297-306, 1992) was employed. This assay is also being used to screen genotoxic activities of other types of compounds (Raabe et al., *Mutation Res.*, in press). I have also continued studies aimed at gaining a better understanding of the mechanisms of action of the plasmacytomagenic agent pristane (2,6,10,14-tetramethylpentadecane). The work has included experiments on the interaction of pristane with model membranes (Janz et al., *Cancer Biochem. Biophys.* 13:85-92, 1992) and is currently being extended to study possible modulation of the activity of protein kinase C (PKC).

Detection of recombinations between IgH and c-myc by PCR (in collaboration with Jurgen Muller). Virtually all murine plasmacytomas (PCTs) carry chromosomal translocations that activate c-myc. The predominant (~90%) c-myc activating chromosomal translocation in pristane-induced PCTs is a reciprocal translocation T(12;15) in which an immunoglobulin heavy-chain switch sequence is joined to the 5' region of c-myc. The most common switch region involved is S<sub>2</sub>. We developed a direct polymerase chain reaction (PCR) method to screen for recombination between c-myc and S<sub>2</sub>. The critical step in establishing the method (collaboration with John Shaughnessy) was the cloning and sequencing of the 5' flank of C<sub>H</sub>, a region with a smaller number of switch repeats than the highly repetitive S<sub>2</sub> region. In applying this PCR method, we detected translocation-specific junction fragments in transplanted (10/16, 63%) and primary (5/15, 33%) plasmacytomas. Moreover, the sensitivity of a nested version of the technique allowed us to discern Ts(12;15) in BALB/c mice in the preneoplastic stage of plasmacytomagenesis (8/20, 40%), as early as 30 days after administration of pristane. We conclude that the T(12;15) is probably a primary, if not initiating, oncogenic step in plasmacytomagenesis. Furthermore, the experimental system has the potential to generate an unlimited number of recombination joints from different cells which could provide a data base for better understanding of the genetic mechanism of c-myc activating chromosomal translocations. The results have recently been discussed at two meetings and are currently being prepared for publication.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 CB 08727-15 LG
PERIOD COVERED October 1, 1992 to September 30, 1993		
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)		
PI:	J.F. Mushinski	Section Chief LG, NCI
Others:	K. Huppi	Expert LG, NCI
	J. Hanley-Hyde	Senior Staff Fellow LG, NCI
	H. Mischak	Visiting Fellow LG, NCI
	E.M. Weissinger	Visiting Associate LG, NCI
COOPERATING UNITS (# any) I.Magrath, K.Bhatia, PB,NCI; J.H.Pierce, CIP,NCI; M.Kazanietz, P.Blumberg,CCTP,NCI; W.Kolch, GSF, Munich, Germany; K.Brown, U.K.Siebenlist, DIR, NIAID; D.Cooper, Univ.S.Fla, Tampa, FL; W.F.Davidson, LG,NCI; J.Hochman, M.Bergel, Hebrew Univ, Jerusalem; Y.Eyler, A.Lewis, NIAID		
LAB/BRANCH	Laboratory of Genetics	
SECTION	Molecular Genetics	
INSTITUTE AND LOCATION	NCI, NIH, Bethesda, MD 20892-0037	
TOTAL MAN-YEARS:	10.0	PROFESSIONAL: 4.2 OTHER: 5.8
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/>	(a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues
<input type="checkbox"/>	(a1) Minors	<input type="checkbox"/> (c) Neither
<input type="checkbox"/>	(a2) Interviews	
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Our research goal is to understand the genes whose altered structure or expression play critical roles in malignancy, cell growth and normal differentiation. We are concentrating on the study of the expression of a group of known oncogenes: abl, bcl-2 and myc, as well as potential oncogenes: Pvt-1, protein kinases C (PKC) and cyclins, in mouse hematopoietic tissues and tumors. Deregulated expression of myc secondary to chromosomal translocations has been shown to be one essential element in the series of genetic alterations that are involved in oil-induced plasmacytomas in BALB/c mice. Some of these myc-activating translocations occur 200-300 kb 3' of c-myc in a region designated Pvt-1. We have shown that Pvt-1 and c-myc are co-amplified in a series of B-cell lymphomas and expressed at a very high level. Pvt-1 expression utilizes alternative splicing of numerous exons, including one that may be as far as 1 Mb 3' of c-myc. We have recently identified three areas of structural similarity between the Pvt-1 genes of mouse and man. This evolutionary conservation suggests strongly that Pvt-1 is a gene of considerable importance to normal cellular metabolism.</p> <p>The ABL-MYC retrovirus, that expresses v-abl and c-myc, rapidly induces i.p. plasmacytomas in BALB/c mice. This transformation also works in vitro if the virus is used to infect suspensions of lymphoid cells that are subsequently transplanted into syngeneic mice. This works even if the cells are early B lymphocyte precursors, i.e., pro-B or pre-B cells. If the mice are immunized before induction of plasmacytomas, 50% of them develop tumors that produce antibodies that are directed toward the immunogen. This technology has been used to produce monoclonal antibodies to several protein and peptide antigens. These results suggest that the most efficient B-cell transformation by ABL-MYC occurs in mature, committed B lymphocytes.</p> <p>Eight isozymes of the PKC family have been shown by us to be expressed in a cell-type specific fashion in hematopoietic cells and cell lines. Expression vectors that stably overexpress all of these isoforms have been prepared. Overexpression of certain of these PKCs in NIH3T3 cells has proved to be transforming in vitro and tumorigenic in nude mice. Similarly, overexpression of these PKC isoforms in a mouse myeloid cell line, 32D, has been shown to impart to these cells the ability to differentiate into macrophages when exposed to phorbol esters.</p> <p>Cyclins B1, D1, D2 and D3 have been cloned and used to identify the chromosome that bears these genes. Expression of these cyclins in hematopoietic tumors has been shown to be cell-type specific.</p>		

### I. Expression of *c-myc* in Three Atypical Plasmacytomas:

1. ABPC 22 and RFPC 2782 are two of the few BALB/c plasmacytomas in which *c-myc* expression is dysregulated in the absence of chromosomal translocation or *v-myc* overexpression. We have determined that both these tumors have dysregulated *c-myc* expression secondary to retroviral integration just 5' of *c-myc*, and in both cases the proviruses are oriented in the opposite transcriptional direction from that of *c-myc*. This finding represents the first examples of mouse B-cell tumors in which *c-myc* was activated by retroviral insertion of an exogenous enhancer. One of the two 72-bp repeated sequences in the typical M-MuLV enhancer is missing in the ABPC 22 provirus, leading to a 65% reduction in the level of *c-myc* expression compared to that of RFPC 2782. This lower level of *c-myc* expression is unique to this plasmacytoma, but this level, expressed constitutively, is sufficient to maintain the transformed state.

2. ABPC 60 has *c-myc* expression dysregulated by a T(12;15) translocation, but this translocation does not interrupt the *c-myc* locus. We have shown that the chromosome 15 breakpoint occurs in the Pvt-1 region, 240 kb 3' of *c-myc*. This arrangement is unique in two ways: 1) *c-myc* and *Sa* are juxtaposed in a head-to-tail configuration; and 2) the T(12;15) interrupts the Pvt-1 locus instead of *c-myc*. More work is needed to understand how this unusual form of translocation activates *c-myc* expression.

### II. Structure and Expression of mouse Pvt-1:

A small but significant proportion of chromosomal translocations in plasmacytomas interrupt chromosome 15 in a region termed Pvt-1, nearly 260 kb 3' of *c-myc*, somehow resulting in dysregulated expression of *c-myc*. It is unclear how translocation to Pvt-1 can affect *c-myc* expression. Despite the isolation of human and mouse Pvt-1 cDNAs, the identification of a Pvt-1 protein has been difficult because of the inability to establish long open reading frames (ORFs) within Pvt-1 cDNA sequences. In a search for longer ORFs, we have analyzed Pvt-1 cDNAs from two mouse sources; a B-cell lymphoma in which *c-myc* and Pvt-1 are co-amplified, and a plasmacytoma with a T(6;15) translocation that interrupts the Pvt-1 locus. We have now identified a 57-bp stretch of DNA which is consistently found at the amino terminus of alternatively spliced transcripts of mouse Pvt-1. This region, designated Pvt-1a, resides in exon 1 and comprises part of the largest ORF (140 aa) established to date in Pvt-1. cDNAs obtained by RT-PCR from RNAs of T(6;15) plasmacytomas shows that Pvt-1a is spliced directly, and in frame, to Ig-C $\kappa$ . Thus Pvt-1a/Ig-C $\kappa$  chimeric proteins could be expressed in these tumors due to the proximity of the potent Ig $\kappa$  enhancer. Construction of Pvt-1/Ig-C $\kappa$  expression vectors as well as the generation of antisera to the Pvt-1a peptide are now in progress to test this hypothesis.

### III. Overexpressed *c-myc* and *v-abl* in the ABL-MYC Retrovirus Rapidly Induces Plasmacytomas.

ABL-MYC is remarkably efficient and rapid in inducing plasmacytomas in adult BALB/c mice. In an attempt to discover the target cell that is transformed by ABL-MYC, we infected suspensions of cells from various lymphoid organs and transplanted them back into syngeneic, but genetically marked, mice for appearance of tumors. Only plasmacytomas arose, even when purified B-cell precursors from fetal liver cells or bone marrow cultures were infected, indicating that very early B lymphocytes could be infected but the overexpression of *c-myc* and *v-abl* forced the cells to differentiate to the plasma cell stage, at which time the transformation potential of ABL-MYC was expressed. Expression of *v-abl* alone (by Abelson virus) blocks differentiation of transformed cells at the pre-B stage, but addition of constitutively expressed *c-myc* seems to abrogate this block. When spleen cells from immunized mice were infected in vitro, plasmacytomas that secreted monoclonal antigen-specific antibodies were produced, suggesting that mature, committed B lymphocytes might be the preferred target for ABL-MYC infection and transformation.

#### IV. Somatic mutagenesis in tumors

1. **p53 Mutations**-Mutation of the p53 tumor-suppressor gene is the most common genetic lesion known in human cancer. 37-70% of Burkitt lymphomas exhibit mutations between exons 5 and 8 of p53. Since mouse plasmacytomas exhibit c-myc-associated chromosomal translocations similar to those of Burkitt lymphomas, we looked for p53 mutation in mouse plasmacytomas and found a surprisingly low (3.4%) incidence of mutation. Thus, the plasmacytoma represents a unique model for the study of alternative mechanisms of p53 inactivation. One candidate, the Mdm-2 gene product, is known to form stable complexes with the wild-type p53 protein and to inhibit p53-mediated transactivation. Thus, disruption of Mdm-2 expression could provide one alternative means of de-stabilizing p53. To examine this possibility, we are currently studying the expression of the Mdm-2 gene in mouse plasmacytomas.

We have also been following p53 mutations in clones of S49, a highly malignant mouse T-cell lymphoma. The solid lymphoma has a mutation in p53 (exon 7, aa 246). A variant subline (T-25Adh), that is not malignant, no longer shows the aa 246 p53 mutation. Instead, a new mutation at aa 242 was identified, and this appears to be associated with the non-malignant phenotype. In a related experiment, we have also been able to associate mutation of the p53 gene (in exon 5) with a malignant or non-malignant phenotype in an adenovirus-infected system. Present studies are focused at generating constructs of mutant p53 that may alter the phenotype of the S49 lymphoma variants or the adenovirus-infected cells.

2. **c-myc mutations**-Although c-myc deregulation was first associated with chromosomal translocations in B-cell tumors, c-myc has also been shown to induce apoptosis in several systems, a function apparently inconsistent with its role in cellular proliferation. We wondered whether a dysfunctional, e.g., mutated c-myc protein, may be present in B-cell tumors. Consistent with this hypothesis, we have identified mutations within the coding region of c-myc in a significant percentage of a large panel of human and mouse tumors. Plans are to extend this survey to other forms of cancer and to construct mutants of c-myc for expression studies.

#### V. Protein Kinase C:

1. **Mouse PKC- $\zeta$**  has been cloned, sequenced and overexpressed in baculovirus-infected insect cells. Our data show evolutionary conservation of the unusual feature of this PKC isoform, the lack of one of the two cysteine-rich "zinc fingers" that are found in all the other PKC isoforms and the inability to bind and be activated by phorbol esters. Expression of PKC- $\zeta$  is unusual, too, in that it appears not to be expressed in normal B and T lymphocytes, but, instead, it is expressed in B- and T-lymphocytic neoplasms. This suggests that expression of this PKC isoform may be involved in lymphomagenesis. We plan to test this notion by constructing a PKC- $\zeta$ -overexpressing retrovirus that can be used to infect intact mice and cultured B and T lymphocytes.

2. **Mouse PKC- $\theta$**  We have cloned the cDNA for mouse PKC- $\theta$  and used it as a probe to study its expression in normal and neoplastic mouse tissues. The literature indicates that PKC- $\theta$  is predominantly expressed in skeletal muscle and hematopoietic cells. Our data confirm that skeletal muscle does express this isozyme abundantly, but the message for this isoform is even more abundant in testes. A Northern study of RNAs from an extensive series of mouse hematopoietic neoplasms indicates that normal T cells and neoplasms of this cell type usually express PKC- $\theta$  abundantly, but this message is barely detectable in normal or neoplastic B lymphocytes and myeloid cells. It is possible that differential expression of this PKC isoform is important at the stage in lymphocytic development when the choice is made between progression along the B or T cell lineage.



3. **PKC- $\epsilon$  and PKC- $\eta$  are proto-oncogenes.** Expression vectors that overexpress all the PKC isoforms were introduced into NIH3T3 cells. Only PKC- $\epsilon$  and PKC- $\eta$  overexpressers caused transformed foci in vitro and grew as tumors in nude mice. Overexpression of PKC- $\delta$ , on the other hand, appeared to induce slowing of growth of NIH3T3 cells.

4. **PKC- $\alpha$  and PKC- $\delta$  are involved in phorbol ester-induced myeloid differentiation.** Expression vectors that overexpress all the PKC isoforms have been introduced into 32D, a murine promonocytic cell line. Only PKC- $\alpha$  and PKC- $\delta$  were able to induce this cell line to differentiate into functional macrophages after treatment with phorbol esters. This indicates that, despite their overall similarities, the different PKC isoforms have different, specific functions in specialized cells.

5. **PKC isoforms translocate to different organelles.** We have overexpressed all the PKC isoforms in NIH3T3 and 32D cells and activated the kinase activity of these abundant proteins by treatment with phorbol esters. Using fluorescence-tagged PKC isoform-specific antibodies, it has been possible to follow the translocation of the individual PKCs to different subcellular sites. Certain PKCs appear targeted to the Golgi apparatus; others associate with the nuclear membrane; and still other isoforms appear to specifically go to intracellular contact points on the cell membrane. We interpret these data as indications of the intracellular sites where the individual isoforms find their specific substrates for phosphorylation.

#### VI. Cyclins.

We have obtained nearly full-length cDNA clones of mouse cyclins B1, D1, D2 and D3 and used them as probes. The chromosomal location of the active (structural) and inactive (pseudogene) genes that encode these proteins have been determined. Expression of these cyclin mRNAs has been found to be tissue-specific in normal cells, and there are indications that transformation is associated with overexpression of certain cyclin transcripts. To test this assumption, the cyclins have been cloned into overexpressing retroviral vectors. These will be used to infect intact mice and cultured lymphoid cells to see if any lead to transformation. Overexpression of these cyclins is also being used to test the hypothesis that the D cyclins play a role in the function of the Rb gene product. Overexpression of cyclin B1 in bacteria and insect cells is yielding abundant amounts of protein suitable for crystallization and 3-D structure determination.

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3. Bhatia KG, Gutierrez MI, **Huppi K**, Siwarski D, Magrath IT. The pattern of p53 mutations in Burkitt's lymphoma differs from that of solid tumors. *Cancer Res* 1992;52: 4273-4276.
4. Boswell CM, Irwin DC, **Goodnight J**, Stein KE. Strain-dependent restricted VH and VL usage by anti-bacterial levan monoclonal antibodies. *J Immunol* 1992;148: 3864-3872.
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7. **Hanley-Hyde J,** Cyclins in the cell cycle: An overview. *Curr Top Micro Immunol* 1992;192: 461-466.
8. **Hanley-Hyde J, Mushinski JF, Sadofsky M, Huppi K, Krall M, Kozak C, Mock B.** Expression of murine cyclin B1 mRNAs and genetic mapping of related genomic sequences. *Genomics* 1992;13: 1018-1030.
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12. **Klinman DM, Dellacqua DK, Conover J, Huppi K.** VH family utilization by IgG anti-DNA-secreting lymphocytes derived from autoimmune MRL-lpr/lpr mice. *Arthritis Rheum* 1993;36: 561-568.
13. **Kunimoto DY, Sneller MC, Claflin L, Mushinski JF, Strober W.** Molecular analysis of double isotype expression in IgA switching. *J Immunol* 1993;150: 1338-1347.
14. **Mischak H, Goodnight JA, Kolch W, Martiny-Baron G, Schaechtle C, Kazanietz MG, Blumberg PM, Pierce JH, Mushinski JF.** Overexpression of protein kinase C-delta and -epsilon in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. *J Biol Chem* 1993;268: 6090-6096.
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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08950-11 LG

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Molecular and biological basis of immune recognition

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

S. Smith-Gill	Senior Investigator, P.I.	LG, NCI
A.H. McDonald	Senior Staff Fellow	LG, NCI
C.R. Mainhart	Microbiologist	LG, NCI
J. Tung	Microbiologist	LG, NCI
M.A. Newman	Biologist	LG, NCI

COOPERATING UNITS (if any)

A.B. Hartman, Dept. of Biologics, Walter Reed; D.R. Davies, E. Padlan, LMB, NIADKD;  
 J.F. Kirsch, Univ. of Cal., Berkeley; S. Sheriff, Squibb Inst. for Med. Research

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

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2.4

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 unreduced type. Do not exceed the space provided.)

Protein-protein interactions underlying molecular recognition are studied, utilizing monoclonal antibodies (Mabs) specific for the protein hen egg white lysozyme (HEL), a protein which has long served as a prototype for investigating the specificity of immune recognition. The X-ray structures of HyHEL-5 Fab complexed with 2 single site-directed mutants of HEL have been solved and refined: R68K which reduces the affinity of the complex by a factor of over 10 to the 3rd power, and R45K, which reduces affinity by only 10-fold. These results represent the first time structures have been obtained for 3 antigens, differing at only a single critical residue, complexed to the same antibody, and will provide valuable insight about the role of Arginine side chains in protein-protein interactions. We are beginning to define fundamental principles that will allow prediction of function from structure, principles that are critical to such applications as antibody design and vaccine development. We are also approaching the problem of vaccine development by investigating immunogenicity and protective epitopes in Shigella flexnerii. In addition, experiments with specific-pathogen-free and conventional BALB/c mice have established that plasmacytoma-refractory SPF BALB/cAnPt mice have naive T cell responses as well as associated restricted B cell responses. These results suggest a significant influence of antigenic exposure, particularly viral antigens, on development of the specificity repertoire and plasmacytomagenesis.

## Major Findings

- A. Molecular Basis of Immunological Recognition in Antibody-Protein Interactions - Dr. S. Smith-Gill, Mr. M.A. Newman, in collaboration with Dr. K. Huppi

The purpose of this research is to define as precisely as possible the molecular basis for specificity and affinity in receptor-ligand interactions. To this end we have utilized as a paradigm monoclonal antibodies (mAbs) specific for the well-defined protein, hen egg white lysozyme (HEL). The majority of secondary response mAbs specific for HEL recognize epitopes that are grouped into larger, nonoverlapping antigenic regions with definable functional boundaries. The apparent antigenic regions show a rough correspondance with the tertiary structure of HEL. Among these late-response antibodies recognizing the defined antigenic domains, there appear to be contrasting structural strategies to recognition of a defined antigenic region.

A series of antibodies functionally related to the structurally defined HyHEL-10 all appear to use very similar structural solutions for recognition of the same epitope; HyHEL-10, HyHEL-8 and HyHEL-26 all utilize members of the same VH and VL gene families (VH 36-60 and VK 23), and X-ray structures of Fab:HEL complexes suggest identical epitopes are recognized (within the limits of resolution of the X-ray structures). This is very similar to the immune responses to many haptens, where a restricted number of VL-VH families are expressed. Ms. A. Aggarwal is characterizing several additional antibodies in the HyHEL-10 group to test the hypothesis that the structural solutions for complementarity to a given epitope are limited.

In contrast, a series of mAbs recognizing a complex region including the epitope recognized by HyHEL-5 appear more variable, and this suggests a variety of structural solutions to a similar epitope. Mr. Newman is PCR cloning and sequencing a group of 7 mAbs whose epitopes overlap that recognized by HHS, but may not be as closely coincident as those recognized by the HyHEL-10 series. To date, at least 2 heavy chain VH families, VH36-60 and VHJ558, are expressed by these functionally related mAbs.

We are continuing detailed analyses of Fab-HEL interfaces utilizing x-ray crystallography (in collaboration with Dr. David Davies, NIAID, Dr. Eduardo Padlan, NIAID, and Dr. Steven Sheriff, Bristol-Meyers Squibb) and by site-directed mutagenesis of both antibody and antigen (in collaboration with Dr. Jack Kirsch, UC Berkeley). Dr. Davies' laboratory has successfully solved the x-ray structure of HyHEL-5 complexed with lysozyme containing a single site mutation R68K, which reduces affinity of the complex by a factor of over  $10^3$ . This will be the first structure of a mutated antigen in complex with an antibody generated to the unmutated, native antigen. Analysis of this structure indicates that a molecule of water replaces the "hole" created by reducing the side chain volume by replacing Arg with Lys and that this water molecule effectively prevents any salt links between residue 68 and heavy chain Glu residues, which are critical to the affinity of the native complex. The x-ray structure of HyHEL-5 complexed with the R45K mutant, which reduces affinity by less than 10-fold, has also been solved, and is currently being

refined. These studies are yielding important information concerning the role of Arginine side chains in protein-protein interactions.

An additional important question is whether receptor ligand binding induces conformational changes in either moiety, and how large or subtle changes which may accompany complex function may underly signal transduction. Antibody-antigen complexes provide an important paradigm for this question, but to date there is only one antibody which both complexed and uncomplexed forms have been structurally defined. Recently, the laboratory of Dr. Eduardo Padlan has solved the structure of uncomplexed HyHEL-10 and it is currently being reported. This will allow comparison with complexed HyHEL-10 which we published in 1988.

- B. Model Systems for Vaccine Development - Dr. Smith-Gill, Mr. Mainhart, in collaboration with Dr. Antoinette Hartman (Department of Biologics Research, Walter Reed Army Institute of Research)

In order to apply principles deriving from our studies on the HEL model system, we are collaborating with Dr. Hartman to investigate the nature of the protective immune response to Shigella flexnerii. The long-term goals of this project, in which Mr. Charles Mainhart is taking a leadership role, include: (i) to test mAbs specific for LPS and other Shigella antigens for ability to confer protective immunity when administered passively to guinea pigs; (ii) to identify the determinants recognized by any protective mAbs; (iii) to "humanize" any mAbs which confer strong protective immunity in an animal model for possible clinical trials. Utilizing a novel route of immunization, we have developed 4 serotype-specific murine mAbs (3 IgM and 1 IgG) recognizing Shigella LPS. These mAbs agglutinate live Shigella. Experiments are in progress to determine if any of these mAbs will inhibit *in vitro* invasion, and whether they can confer protective immunity when administered passively using a guinea pig model developed by Dr. Hartman for vaccine testing and studying the immune response to this pathogen. In addition, we are PCR cloning the genes for expression as IgA, to facilitate passive immunization for mucosal immunity, and for eventual "humanization" if animal model trials are successful.

- C. The Influence of Antigenic Exposure on Immunopathological Responses -- Dr. Ann McDonald [formerly of the Laboratory of Genetics, currently at Milwaukee College of Medicine (3/1/93)], in collaboration with Dr. Linda Byrd (formerly of the LG, currently in Dr. Nash's lab at NIAID/NIH) and Dr. Konrad Huppi.

Dr. Ann McDonald, in collaboration with Dr. Byrd and Dr. Potter, demonstrated that plasmacytoma-susceptible BALB/cAnPt (BALB/c) mice converted to Specific Pathogen-Free (SPF-BALB/c) status have a decreased incidence of pristane-induced plasmacytomas (PCT). Flow cytometry analysis of granuloma cells suggested that plasmacytoma formation is dependent upon exogenous, T-dependent (possibly viral) antigenic stimulation, and that minimal gut flora which was similar in both CON- and SPF-BALB/c mice is not sufficient to render SPF-BALB/c mice susceptible to PCT induction. In order to test this hypothesis, Dr. McDonald examined the immune status of SPF and CON BALB/cAnPt mice by assessing the ability of SPF-BALB/c mice to respond to immunologic challenge

with the soluble antigen, hen egg white lysozyme (HEL) as a means of evaluating differences in T and B cell function. The results establish that the plasmacytoma-refractory SPF BALB/cAnPt mice have naive T-cell responses, which probably contributes to a similarly naive B-cell response.

When cultured in vitro for 5 days with HEL, HEL-primed lymph node cells from SPF-BALB/c mice either failed to proliferate or proliferated to a significantly lesser extent when compared to CON-BALB/c lymph node cells. These results were true over a wide range of immunization concentrations (30-500 ug HEL/mouse). In contrast, HEL-primed spleen cells from SPF-BALB/c mice proliferated as well or better than CON-BALB/c spleen cells when cultured with HEL. Moreover, CON-A proliferative responses were greater in SPF cells than in CON cells. However, antigen-driven production of IFN- $\gamma$  and IL-5 was significantly lower in both SPF spleen and lymph node cells. This reduction in HEL-driven proliferation and cytokine secretion could not be attributed to a lack of colonization of secondary lymphoid organs since flow cytometric analysis of spleen and lymph node cells revealed no difference in the number of recoverable cells and the proportion of lymphocyte subsets (CD4+, CD8+, and CD45+ cells) obtained from SPF- and CON-BALB/c mice. Yet, only CON lymph node cells could be induced to express higher levels of CD44 when cultured with HEL or CON-A. These results suggest that T cells from SPF-BALB/c mice are immunologically immature or naive compared to CON-BALB/c mice. B-cell response, as measured by serum antibody and hybridomas, indicated a much more restricted but also more specific response by the SPF mice, consistent with a naive Th population. In addition, SPF mice had a higher IgM anti-HEL response. Therefore, the lack of infiltrating and/or proliferating Th cells in the peritoneum of pristane-primed SPF-BALB/c mice may be a direct result of antigen deprivation which resulted in fewer memory cells capable of promoting the inflammatory response required for PCT development.

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Kam-Morgan LNW, Kirsch JF, Lavoie TB, Smith-Gill SJ. Site directed mutagenesis as a tool in the analysis of protein-protein interactions. Meth Enzymol 1993;in press.

Benjamin DC, Williams DC, Smith-Gill SJ, Rule GS. Long range conformational changes in a protein antigen due to antigen-antibody interaction. Biochem 1992;31:9539-45.

Newman MA, Mainhart CR, Mallett CP, Lavoie TB, Smith-Gill SJ. Patterns of antibody specificity during the BALB/c immune response to hen egg white lysozyme. J Immunol 1992;149:3260-72.

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McDonald AH, DeGrassi A. Pristane induces an indomethacin inhibitable inflammatory influx of CD4<sup>+</sup> T cells and IFN- $\gamma$  production in plasmacytoma-susceptible BALB/cAnPt mice. Cell Immunol 1993;146:157-70.

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

PROJECT NUMBER

Z01 CB-08952-07 LG

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October 1, 1992 to September 30, 1993

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

Role of the c-myb activation during induction of promonocytic leukemia in mice

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

P.I.:	L. Wolff	Senior Investigator	LG, NCI
	R. Koller	Biologist	LG, NCI
	V. Nazarov	Visiting Fellow	LG, NCI
	J. Bies	Visiting Fellow	LG, NCI

COOPERATING UNITS (if any)

M. Sitbon, Hopital Cochin, Institute National de la Sante et de la Recherche Medicale U152 Paris; H. Fan, Department of Molecular Biology and Biochemistry, University of California, Irvine, CA

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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B

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

The laboratory has focused on two major areas of study. One area involves a determination of the mechanisms involved in development of promonocytic leukemia in our murine model. This disease is induced by a combination of Moloney MuLV or Amphotrophic MuLV and pristane. Our previous data had shown that c-myb activation by insertional mutagenesis in our model system occurs early in bone marrow and spleen within the first month following virus inoculation. This was determined using a sensitive RT-PCR technique to detect leukemia specific gag-myb mRNA. This year we determined that 1) cells undergoing c-myb activation during the preleukemic period can develop as separate clones at multiple bone marrow sites, 2) some mice that are resistant to disease are unable to support virus replication, as in the case of C57BL/6 mice. Alternatively mice of other resistant strains, for example NFS and C3HeJ, are able to support replication and support c-myb activation, but are blocked in tumor formation at a subsequent unknown step, 3) MuLVs that are nonpathogenic in susceptible strains of mice are capable of integrating at the c-myb locus and forming gag-myb mRNA through a splicing event identical to that utilized by Moloney MuLV; this suggests that, in mice infected with these viruses, the block to leukemia development also occurs at a late stage in the disease process. The second major area of study is one that was initiated this year and focuses on the structural-function relationships of the c-myb oncogene product. Sequences encoding mutated or variant forms of Myb protein analogous to the ones expressed in promonocytic tumors as well as the normal c-myb product have been placed in retroviruses vectors. These vectors have been shown to efficiently produce protein in transfected fibroblasts. Experiments aimed at determining the ability of these different forms of Myb to transform cells in vivo and block differentiation are underway.

## MAJOR FINDINGS

### Promonocytic leukemia model.

One major area of ongoing research in the laboratory is an investigation of the molecular as well as physiological requirements for development of disease in our promonocytic leukemia model. Leukemia is induced by a combination of intravenous inoculation of retrovirus and intraperitoneal injection of pristane. It has previously been demonstrated that the role of the replication competent murine leukemia virus (MuLV) is to activate *c-myb* by insertional mutagenesis. It was also demonstrated that this protooncogene activation occurs early after virus infection in the bone marrow and spleen and, therefore, probably accounts for the earliest step in a multistep disease process. The role of pristane is not known although it produces an extensive granuloma from which tumor tissue emerges in the acute phase of disease and it causes an increase in myeloid precursors in the spleen. It was observed that pristane only slightly increases the frequency by which *c-myb* is activated during the preleukemic period.

Clonal nature of *c-myb* activation. Our ability to examine *c-myb* activation during the preleukemic phase of disease depends on the use of a sensitive reverse-transcriptase PCR technique (RT-PCR). It can detect a low number leukemic cells (one in a million) expressing specific *gag-myb* messenger RNA. This year, in an effort to further understand the nature of *c-myb* activation in the preleukemic period, we have examined multiple bone samples from each individual mouse to determine if preleukemic cells develop locally or if they immediately enter into the circulation and repopulate other bone marrow sites. It was observed that individual bone marrow tissues samples from the same mouse were not identical in their expression of specific *gag-myb* products. Sometimes one of two or four sites were positive for *gag-myb* message where as the other sites were not. In addition different size PCR products were often found at different bone marrow sites. This indicated that different bone marrow sites had distinct cell populations since different size products represent different locations of integration within the *c-myb* locus.

### *C-myb* activation in susceptible versus resistant strains of mice.

Although BALB/c and DBA/2 mice are susceptible to disease, seven other strains we have tested are resistant. Three strains C57Bl/6, NFS, and C3H/HeJ mice, which are unable to develop disease, have been selected for further studies aimed at determining the resistance mechanisms. The initial goal was to determine if the block to disease development is at the level of virus replication, integration and activation of *c-myb*, or at a later step in the disease process. It was found that adult C57Bl/6 mice are unable to support the replication of Moloney MuLV. Furthermore, as expected, they were unable to support *c-myb* activation in hematopoietic tissues. It was discovered that in NFS and C3H/HeJ mice, virus replication was not inhibited initially (at two weeks post-virus inoculation mice were shown to have fairly high levels of infectious virus ( $10^3$ - $10^4$  infected cells per  $10^6$  bone marrow cells), but, at four weeks, virus had been cleared in the majority of the mice. In spite of this, when the mice were examined at 4 and 8 weeks post virus infection for



*gag-myb* mRNA by RT-PCR, it was possible to detect specific PCR products in the bone marrow, spleen and granulomas of NFS and C3H/HeJ mice. Thirty-three percent of NFS and 50% of C3H/HeJ were positive for *gag-myb* mRNA, compared to 89% of the BALB/c mice. It appears from this study that resistant gene(s) in NFS and C3H/HeJ mice are inhibiting disease progression at a point subsequent to the insertional mutagenic event that activates *c-myb*.

#### C-*myb* activation by MuLVs that do not induce promonocytic leukemia.

Friend MuLV in contrast to Moloney MuLV was previously found to be negative for disease induction in adult susceptible strains of mice. We set out to determine if the inability to induce leukemia was due to an inability to activate *c-myb*. This was a particularly interesting question since the *gag* region of Moloney MuLV is involved in splicing to *myb* during activation of the protooncogene locus and Friend/Moloney reciprocal recombinant viruses containing *gag* sequences derived from Friend MuLV were also poorly pathogenic in our model system. Mice were injected with pristane and with Friend MuLV, or recombinant viruses having Friend *gag*; at 4 and 8 weeks post-virus infection, the bone marrow, spleen, and granuloma tissues from each mouse were examined for *gag-myb* mRNA. All virus-inoculated mice were positive and every tissue was positive, however, no individual mouse was positive in all tissues.

#### The *c-myb* oncogene.

Previously we had shown that *c-myb* can be activated by insertional mutagenesis in our promonocytic leukemogenic model and that activation can occur in several different ways. With integration at the 5' end of the locus, insertions have been observed in intron 1, 2 or 3 and, in each of these instances, the virus LTR provides promoter/enhancer function, thus bypassing the control from the *c-myb* upstream region. A further consequence of these integrations is the truncation of the proteins at the amino terminus by 20, 47, or 71 amino acids. With integration at the 5' end of the locus, the protein is truncated at the carboxy terminus by 284 amino acids.

The laboratory has now begun a project aimed analyzing the transforming effects of the truncated versions of *c-myb* which are found to be expressed in promonocytic tumors. One goal is to fulfill Koch's postulates by reintroducing mutated forms of Myb in vivo and determining if they can cause disease. A further goal is to determine which changes from the normal gene allow Myb to be more transforming (e.g. truncation at the amino terminus or carboxy terminus or truncation at both ends). In addition, we are looking at the effects of *c-myb* on differentiation of hematopoietic cells. The first step, which is to incorporate sequences for each of these mutated forms into retrovirus vectors, has been completed. This year we obtained sequences encoding the mutated or truncated portions of Myb from tumor cell RNA by PCR amplification. These were recombined with sequences from a normal mouse *myb* cDNA to generate full size *myb* genes (7 versions of the gene altogether resembling those found in rearranged alleles in our tumors or combinations of mutations). These genes were inserted into a retrovirus vector called LXSN. Helper-free viruses have been prepared by transfecting GP86+ENV and GP +E AML2 packaging lines. These viruses when used to infect fibroblasts or

hematopoietic cells have been shown to express the several variants of the gene. This was demonstrated using RT-PCR detection of *gag-myb* mRNA and immune precipitation of Myb protein. These viruses are being tested for their ability to induce leukemia after infecting bone marrow cells from 5FU treated DBA/2 mice and subsequently injecting these cells into irradiated recipients.

Publications:

Nason-Burchenal K, Wolff L. Involvement of the spleen in preleukemic development of a murine retrovirus-induced promonocytic leukemia. *Cancer Res* 1992;52:5317-22.

Mukhopadhyaya R, Wolff L. New sites of proviral integration in murine promonocytic leukemias and evidence for alternate modes of *c-myb* activation. *J Virol* 1992;66:6034-44.

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

PROJECT NUMBER

Z01 CB 08953-03 LG

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

Effects of individual genes on hematopoietic cell differentiation and function

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

P.I.:	W, Davidson	Expert	LG, NCI
	T. Giese	Visiting Associate	LG, NCI

COOPERATING UNITS (if any)

Dr. J. Allison, University of California, Berkeley, CA

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

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 (a2) Interviews

B

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

Mice homozygous for lpr (a defect at the fas locus) and gld develop autoimmunity and profound lymphadenopathy characterized by the accumulation of two functionally anergic T cell subsets, a predominant B220+CD4-CD8- (DN) population and a minor B220+CD4+ population. In the past year we examined the cellular requirements for the accumulation of DN T cells and the nature of the block in signal transduction in these cells. In addition, lpr and gld mice were examined for evidence of abnormalities in T cell deletion in the periphery that may relate to the defective expression of the fas receptor. Depletion of CD8+ T cells in vivo and ongoing adoptive transfer of gld lymphocyte subsets into scid mice revealed that: 1) CD8+ T cells are required for the accumulation of DN T cells; 2) the precursors of DN T cells are present in gld BM, LN and spleen; 3) CD4+ and CD4+B220+ T cells do not spontaneously differentiate into DN T cells; and 4) DN T cells do not grow autonomously in scid mice or differentiate into CD4+ or CD8+ T cells. Treatment of mice with mAb to IFN-gamma and TNF-alpha had no effect on the accumulation of DN T cells but greatly reduced the levels of serum anti-ds DNA Ab. Signal transduction studies showed that DN T cells proliferated and secreted IL-2 in response to TCR crosslinking and Ab-mediated ligation of CD28. This response differed quantitatively and qualitatively from that of normal T cells, suggesting that DN T cells have a higher than normal threshold for stimulation but potentially may be able to proliferate in vivo. Stimulation of lpr and gld mice with SEB confirmed that DN T cells can proliferate in vivo, albeit less efficiently than CD4+ or CD8+ T cells. All T cell subsets expressing V $\beta$ 8 were deleted after SEB treatment but the deletion of lpr and gld CD8+ T cells was impaired. On the basis of these and earlier studies, we propose that DN T cells arise from peripheral, previously activated CD4+, CD8+ or CD4+CD8+ T cells that have down-regulated the expression of CD4, CD8 and the TCR and normally would be deleted by a fas-dependent mechanism. We further propose that the fas receptor does not play a major role in the deletion of self or foreign super Ag-reactive T cells in the thymus or in the periphery.

Major Findings:T cell abnormalities associated with the expression of lpr and gld

C3H mice homozygous for lpr and gld develop strikingly similar diseases characterized by profound lymphadenopathy, autoantibody production and premature death. Lymphadenopathy results predominantly from the accumulation of a unique population of functionally impaired CD4<sup>+</sup>CD8<sup>-</sup>B220<sup>+</sup> (B220<sup>+</sup> double negative, DN) T cells. In addition to these cells, enlarged lpr and gld LN and spleen also contain increased numbers of B lymphocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T cells and another unique population of functionally anergic CD4<sup>+</sup>B220<sup>+</sup> T cells. A high proportion of the CD4<sup>+</sup>B220<sup>-</sup> and CD8<sup>+</sup> T cells in lpr and gld LN have the properties of memory T cells and secrete very high levels of IFN- $\gamma$  and TNF- $\alpha$  following stimulation. Recently, lpr was localized to the fas locus and lpr mice were shown to have an insertion in the second intron of fas that interferes with normal transcription of the gene. In normal mice crosslinking of the fas receptor with Ab induces cell death by apoptosis. The nature of the gld defect is unknown but may involve an abnormality in the expression of the gene that encodes the ligand for the fas receptor. Our studies and those of other investigators suggest that fas is unlikely to play a significant role in the deletion of self Mls or I-E reactive T cells in the thymus. Our current hypothesis is that fas-receptor-mediated cell death is a primary mechanism for deleting antigen-primed T and B lymphocytes in the periphery and, thereby, for regulating the size of the pool of primed or memory T and B cells. This receptor may be particularly important in regulating the numbers of self-reactive T and B cells in the periphery.

Our long term goals are 1) to determine the origin of B220<sup>+</sup> T cells; 2) to elucidate the mechanisms leading to the functional impairment of B220<sup>+</sup> DN T cells; 3) to further characterize the lymphocyte subsets that accumulate and to understand the mechanism leading to their accumulation; and 4) to determine how the various lymphocyte subsets contribute to the development of autoimmune disease. The approaches we have taken over the past year to address these issues are outlined below.

Developmental relationships among T cell subsetsA. Effects of in vivo depletion of CD8<sup>+</sup> T cells in C3H-lpr and C3H-gld mice

Other investigators reported that treatment in vivo with mAb specific for CD4 and IgM prevented the accumulation of DN T cells and greatly reduced the levels of lymphadenopathy and splenomegaly. To determine if CD8<sup>+</sup> T cells play a role in the development of lymphoproliferative disease or autoantibody production, we treated C3H-lpr and C3H-gld with mAb to CD8 for 12 wk beginning at 4 wk of age. These studies showed that depletion of CD8<sup>+</sup> T cells also prevented the accumulation of DN T cells and greatly reduced the level of lymphadenopathy and splenomegaly. Other effects of this treatment included a decrease in the total numbers and an increase in the proportions of CD4<sup>+</sup>, CD4<sup>+</sup>B220<sup>+</sup> T cells and B cells as well as a significant increase in the levels of circulating anti-ds DNA Ab. Although the numbers of CD4<sup>+</sup> T cells and B cells were reduced, they remained higher than the numbers in +/+ mice and accounted for most of the residual lymphadenopathy observed in CD8<sup>+</sup> T cell-depleted mice. The generation of DN T cells, therefore, appears to be a complex process requiring B cells and peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells or possibly CD4<sup>+</sup>CD8<sup>+</sup> T cells. It is not established if DN T cells arise directly from CD4<sup>+</sup> and CD8<sup>+</sup> T cells or CD4<sup>+</sup>CD8<sup>+</sup> T cells, or if these cells only promote the survival of an independent B220<sup>+</sup> DN T cell population. The increase in autoantibody production in CD8<sup>+</sup> T cell-depleted mice may result from the loss of CD8<sup>+</sup>

regulatory T cells or as yet undefined effects of DN T cells on B cells or CD4<sup>+</sup> T cells.

B. Transfer of BALB/c-gld and C3H-gld lymphoid cells into CB.17-scid or C3H-scid mice

In preliminary experiments we established that BALB/c-gld and C3H-gld BM, LN and spleen cells could each transfer lymphoproliferative disease to immunodeficient scid mice. The LN of reconstituted scid mice contained a predominant population of B220<sup>+</sup> DN T cells as well as smaller numbers of CD4<sup>+</sup>, CD4<sup>+</sup>B220<sup>+</sup> and CD8<sup>+</sup> T cells. To determine if individual T cell subsets could differentiate and grow in scid recipients, mice were injected with sorted CD4<sup>+</sup>, CD4<sup>+</sup>B220<sup>+</sup>, B220<sup>+</sup> DN or CD8<sup>+</sup> T cells. In additional experiments, scid mice were reconstituted with combinations of B cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells. To date, these longterm experiments have shown that CD4<sup>+</sup> and CD4<sup>+</sup>B220<sup>+</sup> T cells did not differentiate into B220<sup>+</sup> DN T cells or accumulate in abnormally high numbers. Similarly, B220<sup>+</sup> DN T cells did not differentiate into CD4<sup>+</sup> or CD8<sup>+</sup> T cells and did not grow and accumulate autonomously. Studies with CD8<sup>+</sup> T cell-reconstituted mice and mice reconstituted with mixtures of cells are in progress.

Reversal of clonal anergy by treatment of DN T cells with mAb to the TCR and CD28

B220<sup>+</sup> DN T cells are an enigmatic population that fails to respond to a wide variety of stimuli including crosslinking of the TCR complex, but has many of the hallmarks of previously activated T cells, such as high levels of expression of CD44, LFA-1, CD69 and Ly-6C. One possible explanation for the functional anergy of DN T cells is that they lack the costimulatory signals required to mount a productive immune response. Previously, we reported the lack of expression of one known costimulatory molecule, CD28, on DN T cells. Recently, in collaboration with Dr. Jim Allison, we examined DN T cells for impaired expression or function of another costimulatory molecule, CD28. Studies of mRNA expression and surface antigen expression indicated that lpr and gld B220<sup>+</sup> DN T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressed higher than normal levels of CD28. In costimulation assays, highly purified B220<sup>+</sup> DN T cells cultured with mAb to CD28 in combination with TCR crosslinking and PMA proliferated and secreted low levels of IL-2 and IFN- $\gamma$  but not IL-4 or IL-10. Although DN T cells proliferated and secreted IL-2, these responses differed quantitatively and qualitatively from those of +/+ T cells and lpr and gld B220<sup>+</sup> T cells. Studies of synergism between CD28-, CD69- and Ly-6C-mediated signals revealed that ligation of CD28 enhanced the proliferative response induced by crosslinking of Ly-6C or CD69 on +/+ and lpr and gld CD4<sup>+</sup> and CD8<sup>+</sup> T cells but had no effect on the unresponsiveness of DN T cells to these stimuli. Together, these observations suggest that the mechanisms leading to immunosuppression of DN T cells are complex and appear to involve blockages in signal transduction via the TCR and CD28 and possibly via Ly-6C and CD69 as well.

The inability of DN T cells to respond to TCR crosslinking in the presence of spleen or LN APC from +/+, lpr and gld mice, suggests that normal interactions fail to occur between CD28 and its ligand, B7. One potential explanation for the requirement for Ab-mediated, rather than B7-mediated ligation of CD28 is that the B7/CD28 interaction does not generate a potent enough costimulatory signal to trigger DN T cells. Another possibility is that B7 and CD28 do not interact because the levels of another receptor for B7, CTLA4, are elevated on DN T cells and there is competition between CTLA4 and CD28 for B7. Because CTLA4 has an approximately 20-fold higher affinity for B7 than CD28, increased expression of CTLA4 may significantly influence the transduction of signals by CD28, particularly if the signal delivered by

CTLA4 is a downregulatory one. In preliminary studies to address this question, we observed that lpr and gld DN T cells have higher than normal levels of CTLA4 mRNA transcripts by PCR. Recently, Dr. Allison produced CTLA4-Ig fusion proteins and a mAb that may be specific for CTLA4. In ongoing collaborative studies these reagents are being used to further analyze the interactions between B7, CD28 and CTLA4.

#### The role of cytokines in lymphoproliferative disease and autoimmunity

In a published report we demonstrated that the primed CD4<sup>+</sup> and CD8<sup>+</sup> T cells that progressively accumulate in the lymphoid organs of lpr and gld mice have the potential to secrete high levels of IL-10, IFN- $\gamma$  and TNF- $\alpha$  following stimulation. These cytokines have pleiotropic effects on hematopoietic cells and conceivably could have important secondary influences on the growth or survival of conventional T and B lymphocytes and B220<sup>+</sup> DN T cells. To test this hypothesis we treated C3H-gld mice chronically for 10 wk with anti-TNF- $\alpha$  or anti-IFN- $\gamma$  mAb beginning at 4 wk of age before the onset of disease. These treatments had minimal effects on the accumulation of DN T cells and the severity of lymphadenopathy but did result in a very significant reduction in the levels of circulating IgG anti-ds DNA antibodies. These observations suggest that the accumulation of memory T cells producing high levels of inflammatory cytokines may contribute significantly to the production of autoantibodies in lpr and gld mice. Similar studies are planned with mAb to IL-10.

#### Responsiveness of lpr and gld T cells to superantigen in vivo and in vitro

Superantigens combine with class II MHC to form ligands that bind to particular V $\beta$  elements of the TCR and induce strong proliferative responses. Included within the superantigen family are enterotoxins produced by Staphylococcus aureus. One of these, St. aureus enterotoxin B, SEB, stimulates V $\beta$ 7<sup>+</sup> and V $\beta$ 8<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In vivo these cells proliferate and are then deleted or anergized. Both lpr and gld T cells subsets were treated in vivo and in vitro to evaluate responsiveness to this stimulus. Mice stimulated in vivo also were evaluated for abnormalities in SEB-induced deletion and anergy induction. In vitro studies showed that unlike +/- LN cells, lpr and gld LN cells from 20-30 wk old mice with advanced lymphoproliferative disease, responded poorly to stimulation with SEB. Removal of CD4<sup>+</sup> and CD8<sup>+</sup> T cells reduced the proliferative response to background levels. Further studies with isolated T cell subsets showed that DN T cells were unresponsive to SEB regardless of the age of the animals. This unresponsiveness was not reversed by costimulation with PMA, IL-2 or anti-CD28 mAb. Isolated CD4<sup>+</sup> and CD8<sup>+</sup> T cells from 3-6 wk old lpr and gld mice responded normally to stimulation with SEB but the response declined between 6 and 20 wk of age. Previously we showed that the proportion and total numbers of memory T cells increased between 6 and 20 wk of age, and recently it was reported by others that memory T cells respond poorly to SEB in vitro. Studies are in progress to confirm that the loss of SEB responsiveness by lpr and gld B220<sup>+</sup> T cells correlates with the accumulation of memory T cells. Studies of the in vivo responses of lpr and gld T cells to SEB revealed that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells proliferated and underwent apoptotic death. Interestingly, the rate and extent of superantigen-induced depletion of CD8<sup>+</sup> in both lpr and gld mice was significantly less than that of normal CD8<sup>+</sup> T cells, suggesting that the deletion of CD8<sup>+</sup> T cells in the periphery may be impaired in lpr and gld mice. A modest (2-fold) increase in the numbers of B220<sup>+</sup> DN T cells was observed following treatment with SEB, and this was followed after 1 wk by a return to baseline levels. These data suggest, first, that B220<sup>+</sup> DN T cells may be able to respond, albeit weakly, to antigenic stimulation in vivo, and second, that a fas-dependent mechanism is not required for the deletion of CD4<sup>+</sup> or DN T cells stimulated with

superantigen but may be required for the deletion of CD8<sup>+</sup> T cells. Alternatively, memory CD8<sup>+</sup> T cells may be more resistant to deletion than naive CD8<sup>+</sup> T cells. Experiments are in progress to distinguish between these possibilities.

Our future research will continue to focus on the effects of lpr and gld on T and B cell function and survival. Specifically, we will continue our efforts to understand the complex interactions between B cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the generation and accumulation of DN T cells. For these studies we will use the approaches described above as well as  $\beta$ M and class II knockout mice. Second, we will continue to study the nature of the block in signal transduction that prevents DN T cells from responding to stimuli. In addition, we will begin new studies relating to the effect of lpr and gld on B cell selection, function and survival and also determine if lpr and gld are risk factors in the development of B lineage lymphoid tumors.

#### Publications:

Giese T, Davidson W. Evidence for early onset and polyclonal activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in mice homozygous for lpr and gld. *J Immunol* 1992;149:3097-106.

Kudo A, Thalmann P, Sakaguchi N, Davidson WF, Pierce JH, Melchers F. The expression of the mouse VpreB/1 locus in transformed cell lines and tumors of the B lineage differentiation pathway. *Internatl Immunol* 1992;in press.

Weissinger EM, Mischak H, Goodnight J, Davidson WF, Mushinski JF. Addition of constitutive c-myc expression to Abelson murine leukemia virus changes the phenotype of the cells transformed by the virus from pre-B cell lymphomas to plasmacytomas. *Mol Cell Biol* 1993;13:2578-85.

Giese T, Allison JP, Davidson WF. Functionally anergic lpr and gld B220<sup>+</sup> TCR  $\alpha/\beta$ <sup>+</sup> DN cells proliferate and secrete IL-2 in response to costimulation in vitro with antibodies to CD28 and the TCR. *J Immunol* 1993;in press.





## SUMMARY STATEMENT

### LABORATORY OF BIOCHEMISTRY

DCBDC, NCI

OCTOBER 1, 1992, TO SEPTEMBER 30, 1993

#### INTRODUCTION

The tradition of the Laboratory of Biochemistry is to support diversity and high quality basic research. Fifteen small research groups that address a broad set of independent, but connected, research problems provide the laboratory with a broad and complementary array of technical expertise in molecular biology, genetics, protein chemistry and immunology. During the past year, the research programs established by two new Senior Staff Fellows, Charles Vinson and Mark Mortin, have strengthened the laboratory's expertise in protein chemistry and developmental biology.

The new *Drosophila* genetics facility, organized by Mark Mortin, is now fully functional and utilized by five different groups planning to use genetic techniques in *Drosophila* as a strategy for the functional analysis of proteins *in vivo*.

Protein chemistry and structural biology are becoming the focus of interest of the groups studying the regulation of gene expression. This transition has been facilitated by the purchase of an XLA-ultracentrifuge and a mass spectrometer and the recruitment of post-doctoral fellows well trained in physical and protein chemistry. We continue to take advantage of the unique opportunity at NIH to collaborate with the superb NMR group led by A. Bax, A. Gronenborn and M. Clore (NIDDK) to study the structure of proteins and nucleic acids. A new collaborative project with Ad Bax's group was initiated by Carl Wu.

The scientific environment of the laboratory continues to be stimulating with many productive interactions. Particularly noteworthy this year were the remarkable progress made by Dean Hamer in his new research project on the genetics of sexual dimorphism and the important contributions of Michael Lichten to the elucidation of the mechanism of meiotic recombination in *Saccharomyces cerevisiae*. Major progress was made by Carl Wu and his colleagues in their dissection of the heat stress signal transduction pathway.

#### THE CONTROL OF GENE EXPRESSION

The control of gene expression continues to be the focus of interest of many members of the laboratory.

Carl Wu's group has maintained a focus on gene regulation and chromatin structure, with emphasis on the molecular analysis of transcription factors regulating the heat shock protein (hsp) genes. This group has expanded its efforts in the heat stress signal transduction pathway, in order to capitalize on major advances made over the past year, which included publications in *Science* and *Nature* on the regulation of the heat shock transcription factor HSF. Accordingly, studies of the ftz transcription factors were effectively concluded, and studies on two developmental regulators in *Drosophila* (*FTZ-F1* and *tk*) are being continued by former post-doctoral fellows in their own laboratories. Investigations on the role of chromatin structure on heat shock gene expression were continued by *in vitro* reconstitution techniques, and the ability of the GAGA

transcription factor to assemble an accessible chromatin structure on the heat shock promoter was analyzed, as well as the interactions between the heat shock transcription factor HSF and chromatin. Milligram amounts of HSF protein and protein domains have been produced using bacterial and eukaryotic expression systems, and the purified proteins are being used for physical studies and as biochemical reagents for dissecting the stress signal pathway. The post-translational modifications of HSF that are induced by heat shock are being investigated by means of mass spectrometry.

During the past year Mark Mortin and his collaborators have begun a molecular analysis of mutations in the genes encoding the two largest subunits of RNA polymerase II. A genetic analysis of these mutations had defined a number of phenotypic classes. Sequence data have now shown that mutations in the largest subunit, which interact with the transcription factor Ultrabithorax, map around a central conserved region known as F. Mutations in the second largest subunit, that suppress specific lethal mutations in the largest subunit, map to the conserved region, E. The region surrounding the lethal mutations in the largest subunit and their suppressors in the second largest subunit identify highly conserved domains of RNA polymerase II. Sequence homology between these domains and equally conserved domains of DNA polymerase I led Mark Mortin to predict that all the interacting RNA polymerase II mutations under study map to domains that form a cleft analogous to the cleft in DNA polymerase that is known to bind the double stranded DNA template.

The control of eukaryotic gene expression during development continues to be the focus of attention of Bruce Paterson and his colleagues. They are studying the genes involved in the differentiation of the myoblast during myogenesis. The precursor myoblast is defined early in development within the somite compartment as a group of cells expressing the MyoD family of gene regulatory factors, MyoD, myogenin, Myf5 and MRF4. The expression of these factors not only defines the committed cells but also plays a role in activating the muscle specific genes during terminal differentiation. Dr. Paterson's group has isolated these four genes in the chicken and studied their pattern of expression during development, both *in vitro* and *in vivo*. During the last year, they have concentrated their efforts in determining the role of protein phosphorylation in the regulation of gene expression under the control of these regulatory factors and in the identification of related bHLH proteins that interact with myogenic factors. Dr. Paterson has also taken advantage of *Drosophila* genetics to complement his study of vertebrate myogenesis. He has previously isolated the MyoD homolog in *Drosophila* and is in the process of studying the proteins that regulate its function and of characterizing the upstream and downstream genes of the myogenic pathway in the fly.

The research program of Charles Vinson, who joined the laboratory in February 1991, is centered on the study of protein-DNA interactions. His present work focuses on two DNA binding motifs, the bZIP and the bHLH-Zip motif, motifs for which he has proposed structural models that he is presently testing. Another goal of this group is to identify the specificity rules for leucine zipper dimerization. These rules will allow Dr. Vinson to design dominant-negative molecules that can be introduced into a biological context and disrupt the function of endogenous leucine zipper-containing molecules.

The mouse endogenous retroviral element that encodes the intracisternal A-particle (IAP) continues to be the focus of interest of Edward L. Kuff and Kira Lueders. The factor, EBP-80, previously isolated by Dr. Kuff and his colleagues as an activator of the LTR promoter activity of the IAP element was shown to be identical to the human autoantigen called Ku. Taking advantage of the recent report that Ku mediates the binding and activation of a 350 kDa DNA-dependent protein kinase which phosphorylates the C-terminal domain of RNA polymerase II, Dr. Kuff and his colleagues have demonstrated that the DNA configurations they previously showed to be required

for DNA binding to EBP/Ku, single-stranded gaps, strand separations (bubbles) and stem loop configurations are the same as the ones required for activation of the kinase. They are now examining the possibility that Ku enters the LTR by virtue of a transient strand separation in its specific binding site and then translocates to the nascent transcription complex. Dr. Lueders' efforts are centered on the elucidation of the molecular basis for the selective activation of various members of the IAP gene family. She has also started to use subclass-specific IAP oligonucleotides as multilocus probes for the mapping of the mouse chromosome.

## PROTEINS AND THE CONTROL OF CELLULAR PROCESSES

Claude Klee and her colleagues have concentrated their efforts during the past year on the large scale expression of the two subunits of the calmodulin-regulated protein phosphatase, calcineurin, in *E. coli*. They have demonstrated that myristoylation of the  $\text{Ca}^{2+}$ -binding subunit of the enzyme is required for the enzymatic activity of the holoenzyme. The three-dimensional structure of this subunit, determined in collaboration with Ad Bax and J. Anglister (NIDDK), definitively identified calcineurin B as a member of the "EF-hand"  $\text{Ca}^{2+}$ -modulated proteins. Reconstitution of enzymatically active enzyme from its recombinant subunits will facilitate the crystallization and determination of the structure of the calcineurin-calmodulin complex. The major objective of this group is to elucidate at the molecular level the regulation of this enzyme by  $\text{Ca}^{2+}$  and calmodulin. The recent demonstration by S. Schreiber and his colleagues (Harvard University) that calcineurin is the target of the immunosuppressive drugs emphasizes the need to elucidate the structure of calcineurin to further understand the mechanism of action of the immunosuppressive drugs and to study the role of calcineurin in the  $\text{Ca}^{2+}$ -mediated signal transduction pathway in T cells.

In their studies of the regulation of secretion by  $\text{Ca}^{2+}$  and GTP-binding proteins, Paul Wagner and his colleagues have uncovered a novel mechanism of action of pertussis toxin. They showed that the increased secretory activity following treatment of PC12 cells with pertussis toxin is accompanied by an inhibition of a major cellular protein phosphatase, protein phosphatase-2A. The phosphatase inhibition is independent of any extracellular agonist suggesting that pertussis toxin exerts its effect at a site different from a receptor-coupled G-protein. They are testing the possibility that pertussis toxin modifies a new, and not yet isolated, member of the G-protein family,  $G_e$ , that regulates exocytosis.

Major efforts are being made by Shelby Berger's group to elucidate the role of prothymosin  $\alpha$  in cell division. This nuclear protein, that is absolutely required for cell division, was previously shown to exist as two closely related isoforms derived from alternative splicing. A small percentage of the molecules has been shown this year to be phosphorylated *in vivo* on the amino-terminal serine but in a cell cycle-independent manner. This group is now trying to identify the function(s) of the two isoforms of prothymosin  $\alpha$  and to understand how low level phosphorylation may affect the function of this very abundant protein by modifying its ability to interact with nuclear macromolecules.

Beverly Peterkofsky has a long standing interest in the regulation of the synthesis of extracellular matrix components, collagen and proteoglycans. During the last two years, she has concentrated her efforts on the identification of circulating inhibitors of collagen and proteoglycan synthesis that are induced by vitamin C deficiency and fasting. Definitive identification of the inhibitor as the insulin-like growth factor binding protein (IGFBP)-1 has now been established by removal of the inhibitor from sera of fasted and vitamin C-deficient guinea pigs with specific antibodies raised against IGFBP 1 and 2.

Michael Mage and his colleagues have been successful in synthesizing properly folded and functionally active recombinant single chain Class 1 MHC molecules with the b2 microglobulin covalently linked to the heavy chain. One such recombinant protein has been stably expressed as a cell surface molecule and shown to be functional in antigen presentation. Encouraged by these positive results they are now engineering more complex constructs, encoding covalently linked trimeric molecules consisting of the heavy chain, the b2 microglobulin and an antigenic peptide with the ultimate goal to construct vaccines in which the antigenic peptide is covalently attached to a cell surface class 1 MHC molecule.

## THE ORGANIZATION OF THE HUMAN GENOME

The major clinical and biological implications of the mapping of human genes and DNA sequences onto human chromosomes have long been recognized in the laboratory by Maxine Singer and Wes McBride. They were joined this year by Dean Hamer.

Dr. Singer and her colleagues have been concentrating on the mechanism of translation of the bicistronic mRNA of the LINE-1 human transposable element (LIHs) and on the nature of the translation products. Translation initiation of the first open reading frame to yield p40 appears to involve scanning of ribosomes from 5' of the AUG start codon at base 900; translation of the second open reading frame appears to be initiated by ribosomes attaching *de novo* within the mRNA. Both p40 and polypeptides encoded by the second open reading frame have been expressed in *E. coli* as fusion proteins containing a histidine hexamer, thereby permitting purification on Ni-agarose resin. The leucine zipper protein p40 purified from *E. coli* forms homomultimers which may involve disulfide bonds.

Several new genes have been mapped by Dr. McBride and his colleagues. They include the interleukin 2 gamma receptor gene (IL-2RG) in collaboration with W.J. Leonard, a phosphotyrosine phosphatase gene (PAC) with K. Kelly, the plasma membrane ATPase isoform 3 with E. Carafoli and the transglutaminase genes 2 and 3 with P. Steinert. A tight linkage between the disease locus XSCID and IL-2RG suggested that XSCID is caused by a mutation in IL-2RG. This prediction has been confirmed by Noguchi and Leonard, who showed that three unrelated patients with XSCID syndrome had different mutations in IL-2RG. Another major research project initiated during the past year involves the identification, sequencing, and characterization of DNA sequences containing highly polymorphic short tandem repeats. Ten of these loci, containing triplet repeats, span an interval of 70 cM on human chromosome 22. They provide a useful panel of microsatellites for mapping disease genes on this chromosome.

Dr. Hamer has initiated a new challenging research program. His group is now studying the molecular basis for the sexual differentiation of neural structure and function in humans, *Drosophila*, and rats. Their most striking finding is a linkage between DNA markers on the X chromosome and male sexual orientation. If confirmed, this will provide the most compelling evidence to date that human sexuality is genetically influenced, and will represent a breakthrough in the study of human behavior.

## DNA REPLICATION

This has been a particularly productive year for Michael Yarmolinsky and his colleagues who are studying the mechanism of plasmid maintenance and for Michael Lichten and his colleagues studying the mechanism of meiotic recombination in the yeast *Saccharomyces cerevisiae*.

The group of Drs. Yarmolinsky and Chatteraj have focused on three regions of the DNA of the low copy number plasmid P1 that are critical for its maintenance. Studies of the replication origin have revealed significant conformational differences between the two strands once strand-opening has been induced by the cooperative action of initiator proteins DnaA and RepA. The role of host-encoded chaperone proteins in relation to RepA structure and function is being clarified. Studies of the plasmid centromere-analog, *parS*, have revealed that whether this element increases or decreases plasmid stability is a function of its context and of the concentration of the plasmid-encoded partition protein ParB. Critical domains of *parS* and ParB have been defined by taking advantage of genetic suppression of destabilization. Studies of two P1 genes that constitute an addiction operon have suggested the nature of the mechanisms which assure that the ratio of the gene products (a poison and its antidote) would be biased in favor of the antidote during plasmid retention and in favor of the poison following plasmid loss.

Recent studies of Dr. Lichten and his colleagues have revealed the involvement of chromatin structure in the control of meiotic recombination and open the way to a biochemical study of this complex biological event. They have demonstrated that chromosomal elements as far away as 20 kb from their targets can influence the frequency of meiotic recombination. These elements were also shown to influence in a parallel fashion the frequency of double strand breaks and the accessibility of DNA in chromatin to non-specific endonucleases. Enhancer elements that act as stimulators (in *cis*) of meiotic recombination have been identified, as well as sequences that appear to suppress meiotic recombination. The latter were found to be effective in *trans* as well as in *cis* to the locus of recombination, suggesting that chromosome pairing precedes the initiation of meiotic recombination rather than occurring as a result of such recombination.

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

PROJECT NUMBER

Z01 CB 00366-22 LB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Expression of endogenous retroviral elements as indicators of cellular function

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

E. L. Kuff Chief, Biosynthesis Section LB NCI  
 J. Fewell Microbiologist LB NCI

COOPERATING UNITS (if any)

Dr. Carl Anderson, Biology Department, Brookhaven National Laboratory, N.Y.;  
 Dr. Miriam Falzon, Department of Pharmacology, Univ. of Texas Medical Center,  
 Galveston TX; Dr. Michael Potter, Laboratory of Genetics, DCBDC, NCI

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

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.)

A nuclear factor, EBP-80, purified in our laboratory as a transcription factor for intracisternal A-particle LTRs, has proven to be similar to or identical with a previously recognized human autoantigen, Ku. Ku is known to bind to the ends of linear duplex DNA. We have shown that end-binding is only one example of a more general property of EBP-80/Ku, namely its capacity to recognize and bind to transitions between single and double stranded DNA. Recently, Ku has been shown by others to mediate the binding and activation of a 350 kDa DNA-dependent protein kinase which can phosphorylate functionally important sites in p53 and the C-terminal domain of RNA polymerase II. Using a highly purified kinase preparation provided by Dr. C. Anderson (Brookhaven), together with our own EBP-80/Ku preparation, we have shown that the kinase can be activated by circular DNA containing a bubble of non-homology or a short single-stranded region (gap), and also by a linear construct terminating in two stem-loop configurations. The kinase/EBP-80 complex is not activated by completely duplex closed circular DNA. With Dr. M. Falzon (Galveston), we are defining the minimal and optimal bubble and gap sizes for binding and activation. EBP-80/Ku, by binding to the transcription bubble, may bring the protein kinase into a position where it can carry out the necessary phosphorylation of the RNA polymerase C-terminal domain.

Ku is generally considered to be almost exclusively localized in the nuclei of interphase cells. We have found, however, by both immunostaining of fixed cells and Western blots of cell extracts, that Ku antigen is primarily cytoplasmic in very sparsely grown cells, including HeLa and primary human keratinocytes. The proportion of nuclear antigen increases with cell density until the typical nuclear distribution is observed in confluent cultures. Sparse cells plated onto a monolayer of mouse 3T3 fibroblasts rapidly acquired the exclusively nuclear localization, suggesting that the redistribution of Ku from cytoplasm to nucleus is a response to cell-cell contact.

## Project Description

Major Findings:

## A. Transacting cellular factors for IAP proviral activation (Drs. M. Falzon and V. Morozov)

The nuclear factor EBP-80, isolated by Dr. Falzon from human tumor cell lines HeLa and 293, was shown to stimulate transcription from IAP LTRs in an in vitro system and mediate in part the known inhibitory effect of DNA methylation on LTR promoter activity both in vitro and in transfected cells (see previous Annual Reports). Subsequently, we found that EBP-80 was very similar to, and probably identical with, Ku, a previously recognized human nuclear autoantigen. Ku was known to bind in vitro to ends of duplex DNA but not to closed circular forms. Its function in cells was not known. In last year's Report, we described our observations, now published, leading to the conclusion that EBP-80/Ku recognized transitions between single and double-stranded DNA, including bubbles, single strand gaps and stem-loop structures in closed circular DNAs, and that its end-binding property reflected transient melting and strand separation at the termini of linear duplex DNAs. A model was developed for the reversible binding of EBP-80/Ku to its known target sequence in the IAP LTR: binding was postulated to involve a local strand separation driven by relaxation of DNA supercoiling, and release a methylation-favored B to Z form transition in adjacent DNA which had the effect of inhibiting the strand separation. Bound EBP-80/Ku could exert its effect locally or, through an energy-free translocation process described by others, at more distant locations.

Very recently, several laboratories have reported that Ku associates with a previously recognized 350 kDa DNA-dependent protein kinase (PK350) and mediates the in vitro DNA-binding and activation of this enzyme. PK350 can phosphorylate sites in the C-terminal domain of RNA polymerase II, a function necessary to initiate translocation of the transcription complex. If PK350 indeed fulfills this function, then both it and its associated Ku may be essential components of the complex. Ku could direct PK350 to initiation sites by virtue of its affinity for the single-to-double strand transitions at transcription bubbles.

Currently we are testing for activation of PK350 by linear duplex DNAs and by constructs containing internal regions of strand separation. Purified PK350 and a specific target oligopeptide have been provided on a collaborative basis by Dr. Carl Anderson at Brookhaven. The enzyme preparation as supplied has a DNA-dependent activity which is due to a certain content of contaminating Ku; activity is increased 3-4 fold by saturating amounts of added EBP-80. Using an empirically optimized ratio of EBP-80 to PK350, we have found that activation by the various DNA constructs accurately reflects their binding affinity for EBP-80. Thus, the bubble- and gap-containing minicircles were both as effective as a 34 bp duplex oligonucleotide in activating the enzyme, and somewhat more effective than either the linearized 350 bp minicircle or a 64 bp oligonucleotide. The completely double-stranded minicircle was essentially inactive. These findings lend support to the likelihood that EBP-80/Ku can direct PK350 to positions of internal DNA strand separation.

In continued collaboration with Dr. Falzon, presently at the University of Texas Medical Center in Galveston, we are determining the optimal and minimal sizes of single-stranded gaps and bubbles in terms of their capacity to bind EBP-80 and activate PK350. Thus far, Dr. Falzon has found that bubbles 6 and 3 nucleotides long have much lower binding affinity for EBP-80 than the 30 nt bubble we have previously studied. It will be interesting if the minimal opening required for strong binding is in the order of one DNA helix turn, considered to be the approximate size of eukaryotic transcription bubbles. Ku has recently been reported to bind to single strand nicks introduced into circular DNA by treatment with EcoRI in the presence of ethidium bromide. Minicircle constructs permit the determination of binding affinity for single-strand gaps of precisely known length and sequence. These studies are in progress.

#### B. Growth-related changes in the intracellular distribution of EBP-80 (J. Fewell)

Ku is generally described as a nuclear protein in interphase cells, although there is one report of its association with the plasma membranes of HeLa cells. Immunostaining with monoclonal antibodies has shown that the antigen is dispersed into the cytoplasm of HeLa cells at mitosis and re-accumulates in the sister nuclei in early G1. We have examined the nucleo-cytoplasmic distribution of Ku in several cell types, using both a rabbit polyclonal antibody against recombinant 70 kD subunit and a human antibody immunopurified on authentic Ku and reactive with both the 70 and 86 kD components. Antigen was found to be largely cytoplasmic in sparse cultures of three virus-transformed human cell lines [HeLa (HPV, cervical epithelium), HV-1 (HPV-keratinocytes) and 293 (adenovirus, kidney cells)] and 2 strongly contact-inhibited cell types [CV-1 (monkey kidney) and normal keratinocytes]. Cytoplasmic antigen was detected by immunostaining of fixed cells and protein blots of cell extracts. Ku was also demonstrated in cytoplasts prepared by cytochalasin treatment of CV-1 cells. In each cell type, the proportion of nuclear antigen increased with increasing cell density until in heavy or confluent cultures, demonstrable antigen was almost exclusively nuclear, the situation commonly reported. Both HeLa and 293 cells showed a rapid shift of antigen to an exclusively nuclear position when they were plated as single cells on a confluent monolayer of mouse 3T3 fibroblasts. Our results suggest that the intracellular localization of Ku is responsive to cell-cell contact, and we are currently examining the possibility that it is part of a signaling mechanism through which such contacts could influence the transcriptional activities of the cells.

#### C. Interaction between EBP-80/Ku and IAP LTRs

We are currently developing the molecular reagents for testing the binding model proposed in section A. As a first step, S. Boyd has modified the minicircle-yielding construct of Choy and Adhya by inserting the CAT gene behind a new multicloning site. Mutated forms of the LTR generated by PCR will now be inserted upstream of the CAT gene and tested for binding and promoter activity in the form of excised minicircles. Interference by plasmid sequences is eliminated by this approach. The modified plasmid containing the CAT reporter gene may be a useful reagent for other investigators.



D. Patterns of IAP LTR hypomethylation and expression in primary plasmacytomas of BALB/c mice (see also Annual Report of K. Lueders)

Primary plasmacytomas induced by either virus or pristane have been provided by Dr. M. Potter. DNAs from these tumors show reproducible patterns of IAP LTR hypomethylation, as revealed by both single- and two-dimensional gel electrophoresis. Overall levels of IAP transcripts are not significantly increased over that seen in LPS-stimulated normal B cells; however, a specific variant type of IAP provirus is newly expressed. These observations indicate that IAP hypomethylation follows a specific pattern in early plasmacytomas, possibly reflecting a corresponding modification pattern of cellular genes.

Publications:

Mietz JA, Kuff EL. Intracisternal A-particle specific oligonucleotides provide multilocus probe for genetic linkage studies in the mouse, *Mammalian Genome* 1992;3:447-51.

Falzon M, Kuff EL. The nucleotide sequence of a mouse cDNA encoding the 80 kDa subunit of the Ku (p70/p80) autoantigen, *Nucleic Acid Res* 1992;20:3784.

Lueders KK, Frankel WN, Mietz JA, Kuff EL. Genomic mapping of intracisternal A-particle proviral elements, *Mammalian Genome* 1993;4:69-77.

Falzon M, Fewell JW, Kuff EL. EBP-80, a transcription factor closely resembling the human autoantigen Ku, recognizes single-to-double strand transitions in DNA, *J Biol Chem*, in press.

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

PROJECT NUMBER

Z01 CB 00945-20 LB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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	Chief, Biological Interactions Section	LB	NCI
A. Gosiewska	Visiting Fellow	LB	NCI
S. Wilson	Biologist	LB	NCI
U. Varadharanjan	Guest Researcher	LB	NCI
F. Mahmoodian	Visiting Fellow	LB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Biological Interactions Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

2.5

OTHER:

2

CHECK APPROPRIATE BOX(ES)

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

B

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

Our previous studies provided evidence 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 proteoglycan synthesis. Sera from both groups contain a circulating factor that inhibits these functions and DNA synthesis in cultured connective tissue cells and inhibition is reversed by insulin-like growth factor (IGF)-I. The presence of the inhibitor in sera was associated with an increase in low molecular weight IGF binding proteins (IGFBPs) that can inhibit binding of IGF-I to its cellular receptor. We have identified the binding proteins as IGFBP-1 and IGFBP-2. Antibodies to purified rat IGFBPs 1 and 2 were used to remove the corresponding guinea pig IGFBPs from fasted and scorbutic guinea pig sera. Removal of the IGFBPs also removed the ability of the sera to inhibit IGF-I-dependent functions such as DNA and collagen synthesis. These results unequivocally establish the identity of the inhibitor as IGFBPs. Taken together with our results showing that the IGFBPs and inhibitor are induced in parallel and that induction occurs prior to or concomitant with the decrease in collagen mRNA concentrations in bone, skin and cartilage, our studies suggest that the IGFBPs also function as inhibitors of collagen synthesis during fasting and vitamin C deficiency in vivo.

## 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 (IGFs) and their binding proteins in this regulation.

Major Findings:

## I. Role of IGF Binding Proteins in Regulating Collagen and Proteoglycan Synthesis During Scurvy and Fasting

## A. Background

Our previous results suggested that ascorbate-deficient and fasted (ascorbate-supplemented) guinea pigs are equivalent with respect to the mechanisms by which collagen and proteoglycan synthesis are decreased in connective tissues. Furthermore, sera from these animals could transmit the defects in extracellular matrix synthesis to cultured connective tissue cells in the presence of ascorbate through the action of an inhibitor. These sera also inhibited the stimulation of DNA synthesis in quiescent 3T3 cells. The inhibition of all of these processes was reversed by IGF-I although inhibition occurred whether or not the serum containing the inhibitor had normal or reduced levels of IGF-I. The ability of IGF-I to reverse the inhibition and other results suggested that the inhibitor might be one or more IGF-I binding proteins (IGFBPs).

## B. Identification of the IGFBPs in Guinea Pig Sera

1. 40- and 44-kDa IGFBPs, unchanged by nutritional deficiencies: These IGFBPs migrated in SDS-PAGE like two differentially N-glycosylated forms of IGFBP-3. After treatment with N-glycanase, these bands disappeared and were converted to a single band corresponding to a 36-kDa protein, confirming their identification as guinea pig BP-3.

2. 29- and 35-kDa IGFBPs, induced during nutritional deficiencies: Based on the known masses of the IGFBPs and their affinities for the IGFs, and previous observations that IGFBP-1 is increased in sera of fasted humans and rats, it seemed likely that the induced proteins were BP-1 and BP-2. Immunochemistry with anti-human IGFBP-1 and anti-BP-2 peptides identified these proteins as IGFBPs 1 and 2.

3. Preparation of antibodies to native BP-1 and BP-2: Since we required additional antibodies and there is immunological cross-reactivity between species for both IGFBP-1 and -2, rat cell lines were used to isolate the binding proteins.

4. Inhibition of IGF-I-dependent functions by purified IGFBPs 1 and 2: Addition of purified rat IGFBPs 1 and 2 at 400 ng/ml to 3T3 cells cultured in normal

guinea pig serum inhibited DNA and collagen synthesis, although IGFBP-1 was more effective than IGFBP-2. These results demonstrated that IGFBPs acted similarly to the endogenous inhibitor in FGPS and SGPS.

5. Removal of IGFBPs from guinea pig sera: Guinea pig sera were incubated with antibodies against purified rat IGFBP-1 and/or rat IGFBP-2 and passed through a Protein A-Sepharose column to remove the IGFBPs. A fasted guinea pig serum (FGPS) pool with a 3:1 ratio of IGFBP-1 to IGFBP-2, and a scorbutic guinea pig serum (SGPS) pool with a 2:1 ratio of IGFBP-2 to IGFBP-1 were used. The inhibitory effects of the pass-through fractions from FGPS and SGPS on DNA or collagen synthesis in 3T3 cells were compared to effects of similarly treated normal guinea pig serum fractions. Removal of IGFBP-1 reversed inhibition of DNA synthesis by half and collagen synthesis by 90%, while removal of IGFBP-2 had much less of an effect, in agreement with results for the purified rat IGFBPs that showed IGFBP-1 to be a more effective inhibitor. When both binding proteins were removed, there was almost complete reversal of inhibition of DNA and collagen synthesis. These results provide conclusive evidence that IGFBPs 1 and 2 in FGPS and SGPS are responsible for the inhibition of IGF-I dependent functions by these sera in cell cultures.

#### C. Relationship of IGFBPs to In Vivo Expression of Collagen

This aspect of the project was designed to determine whether the temporal expression of the IGFBPs during nutritional deprivation in vivo was compatible with their proposed role as inhibitors of collagen synthesis.

1. Expression of IGFBPs relative to collagen gene expression: We previously reported that mRNAs for the IGFBPs were induced prior to the decrease in collagen mRNAs in both fasting and vitamin C deficiency. We now have found that the concentrations of circulating IGFBPs 1 and 2 were increased, although the pattern of induction of IGFBP-1 paralleled that of the inhibitor of IGF-I action. These increases occurred prior to the decreases in the expression of the collagen mRNAs in connective tissues.

2. Factors regulating induction of IGFBPs: Since insulin inhibits and glucocorticoids stimulate the expression of IGFBP-1 in cell cultures, we measured the concentration of these factors in sera from guinea pigs during the course of both nutritional deficiencies. Insulin concentrations already were decreased after 10 h of fasting. Changes in cortisol concentrations were biphasic and did not correlate with changes in IGFBP-1. Thus, decreased insulin may lead to induction of the IGFBPs.

#### II. Effects of Vitamin C Deficiency on Wound Healing in Guinea Pigs

##### A. Background

Early work had shown that wound healing was impaired during vitamin C deficiency. It was suggested that this resulted from defective collagen formation due to the requirement for ascorbate in proline hydroxylation. Based on our recent work, however, inhibition of collagen synthesis appears to be related to the appearance of IGFBPs, as discussed above. Phase I of scurvy consists of the first two weeks on the vitamin C-free diet when the guinea pigs grow as well as controls although vitamin C levels are decreased by about

90% after one week. In past studies, wound healing was defective and concentrations of hydroxyproline were markedly decreased even in phase I. It seemed possible that in wound repair tissue, regulation of collagen formation during scurvy might operate via a different mechanism than in non-repair connective tissues. If fibroblasts proliferated rapidly in a wound after ascorbate levels were decreased in the animal, it seems likely that they would have no stored ascorbate, unlike cells in other tissues. In this respect, the wound fibroblasts would be similar to fibroblasts grown in culture medium without ascorbate, where proline hydroxylation is severely inhibited and procollagen secretion is inhibited. This project was initiated to determine whether defective collagen synthesis or proline hydroxylation occurred in wound tissue during phase I of scurvy when collagen synthesis in non-repair connective tissues is not affected.

#### B. Collagen Gene Expression in Implants

Guinea pigs were placed on a vitamin C-free diet with or without oral ascorbate supplementation. After seven days, when ascorbate levels in tissues were decreased, vinyl sponge squares were implanted under the skin to collect granulation tissue and the wound was closed with a wound clip. After 10 days (17 days on the diet), the implants were analyzed. We reported that in the implants from scorbutic guinea pigs, collagen synthesis was reduced by about 40% but proline hydroxylation was normal. Recent experiments show that the concentrations for types I and III collagens were decreased to about the same extent as collagen synthesis.

As expected for this early phase of scurvy, the concentrations of circulating IGFBPs 1 and 2 were not increased and therefore could not be responsible for the decreased expression of collagens in the scorbutic implant. The expression of several cytokines known to modulate collagen gene expression such as TGF- $\beta$ , interleukin-1, and tumor necrosis factor was examined but no differences were observed between implants from normal or scorbutic animals.

#### Publications:

Takeda K, Gosiewska A, Peterkofsky B. Similar, but not identical, modulation of expression of extracellular matrix components during in vitro and in vivo aging of human skin fibroblasts, *J Cell Physiol* 1992;153:450-9.

Schalk EM, Gosiewska A, Prather W, Peterkofsky B. Transcriptional regulation of the pro $\alpha$ 1(I) collagen gene in pro $\alpha$ 1(I)-deficient, chemically transformed Syrian hamster embryo fibroblasts, *Biochem Biophys Res Commun* 1992;188:780-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 05202-26 LB
PERIOD COVERED October 1, 1992 to September 30, 1993		
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.W. McBride	Chief, Cellular Regulation Section	LB NCI
H.-F. Yi	Visiting Fellow	LB NCI
D. Sabourin	Staff Fellow	LB NCI
J. Clark	Laboratory Worker	LB NCI
COOPERATING UNITS (if any) NIAMSD: P. Steinert, S.J. Bale; NCI: K. Kelly, P. Howley, S. Rosenberg, F. Gonzalez; NIMH: S.J. Lolait, M.J. Brownstein; A. Arnold (Harvard); N. Nussmeier (U. Chicago); R. Pirtle (U. Texas); E. Carafoli (Zurich); M. Wang, Oncor, Gaithersburg, MD.		
LAB/BRANCH Laboratory of Biochemistry, DCBDC		
SECTION Cellular Regulation Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.75	2.25	0.5
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 <span style="float: right;">B</span>		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)		
<p>Studies in collaboration with Drs. W.J. Leonard and M. Noguchi (NHLBI) have resulted in localization of the interleukin 2 gamma receptor gene on the human X-chromosome. Mapping in human-rodent somatic cell hybrids combined with in situ hybridization and genetic linkage analysis using single strand conformational polymorphisms within introns of the IL-2RY gene allowed this gene to be localized to band Xq13, and it was shown to be tightly linked to PGK1 and anonymous DNA markers including DXS106. The disease locus for X-linked Severe Combined Immunodeficiency (XSCID) is linked to these same loci, strongly suggesting that XSCID may involve mutations in the IL-2RY gene. This interpretation subsequently has been confirmed by our collaborators (Noguchi and Leonard) who demonstrated that each of three unrelated patients with XSCID has a different mutation in his IL-2RY gene resulting in a different premature stop codon and C-terminal truncation. Further studies are in progress to optimize carrier detection and gene therapy. Cloned genes recently mapped include a phosphotyrosine phosphatase (PAC) with K. Kelly, a plasma membrane Ca<sup>2+</sup> ATPase isoform 3 (PMCA3) with E. Carafoli, and two additional transglutaminases (TGM2 &amp; TGM3) with P. Steinert; about 12-15 additional genes are currently being mapped. A third project has involved identification and sequencing of DNA tracts containing highly polymorphic short tandem repeats. A group of ten of these loci containing triplet repeats have been isolated and they provide a useful panel of microsatellites for mapping disease genes on human chromosome 22 and these ordered loci span an interval of 70 cM on this chromosome.</p>		

## Project Description

Objectives:

1) Human chromosomal mapping of protooncogenes and genes involved in DNA synthesis, carcinogen metabolism, and regulation of cell proliferation and gene expression, and understanding the role of these genes in human neoplasia, 2) mapping the genes for hereditary cancer predisposition syndromes, 3) isolating additional highly polymorphic markers on specific chromosomes for high resolution maps, and 4) developing a map of the human genome and identifying specific genes or gene alterations involved in hereditary diseases.

Major Findings:

A panel of human-rodent somatic cell hybrids previously isolated and characterized in this laboratory continues to be used for chromosomal mapping of cloned human genes in collaboration with investigators at NIH and elsewhere. This panel has been used to localize several additional genes during the past year including the interleukin2 gamma receptor (IL2RY) gene with Dr. W.J. Leonard (NHLBI), a phosphotyrosine phosphatase (PAC) with Dr. K. Kelly (NCI), the plasma membrane  $Ca^{2+}$  ATPase isoform 3 with Dr. E. Carafoli (Zurich), and Transglutaminase 2 (TGM2) and Transglutaminase 3 (TGM3) with Dr. P. Steinert (NIAMSD). About 12-15 additional genes are currently being mapped.

Cloning of a cDNA for human IL-2RY was reported by Takeshita et al. in 1992, and both the genomic sequence and IL-2RY cDNA were subsequently cloned by M. Noguchi & W.J. Leonard and colleagues at NIH. In collaboration with Noguchi & Leonard, the IL-2RY gene was immediately localized to the human X-chromosome by Southern analysis of our panel of somatic cell hybrids using the cDNA probe. The gene also was regionally localized to the proximal portion of the X-chromosomal long arm (Xcen-q13) by analysis of hybrids containing specific X-chromosomal breaks and translocations. This localization was confirmed and further narrowed to band Xq13 by W.S. Modi (NCI) using fluorescence in situ hybridization. Of interest was the fact that the most common form of Severe Combined Immunodeficiency is X-linked (XSCID), and the disease is characterized by profound defects of cellular and humoral immunity due to the failure of T-lymphocytes to proliferate and differentiate. Based upon its X-chromosomal location and function, IL-2RY became a candidate gene for this disease. Genetic linkage analysis was used to further explore this possibility. No RFLPs or microsatellites could be detected at this locus and hence other types of DNA polymorphisms were sought. Oligonucleotide primers were synthesized to permit PCR amplification of 200-400 bp segments of each of the seven introns of this gene. These  $^{32}P$ -labeled PCR products were denatured and analyzed by autoradiography after high voltage electrophoresis in nondenaturing polyacrylamide gels. Single stranded conformational polymorphisms (i.e. SSCPs) were found in two of the introns by this method. These SSCPs were then used for genetic linkage analysis in 40 large CEPH families. Both two-point and multipoint linkage analysis showed that the IL-2RY gene was located at Xq13 and that both XSCID and IL-2RY were tightly linked to the same markers. Noguchi and Leonard then demonstrated by DNA sequencing that each of three unrelated

patients with XSCID has a different mutation in his IL-2RY gene resulting in a different premature stop codon and predicted C-terminal truncation, indicating that XSCID is caused by mutations in IL-2RY. Carrier female detection is under way in family members and in additional XSCID families. Testing of PCR amplified exonic sequences by SSCP and heteroduplex analysis is being evaluated for reliability in detecting mutations in this gene. The results currently indicate that both methods are useful in detecting some, but not all, mutations in this gene. Studies are also in progress directed toward gene therapy of affected males using the cloned gene inserted into an expression vector.

We have localized the PAC gene (K. Kelly, NCI) to the centromeric region of human chromosome 2 by Southern analysis of the panel of somatic cell hybrids including those with specific breaks involving this chromosome and using in situ hybridization. Hybrids prepared from human fibroblasts containing at(2;6)(q11;q15) reciprocal translocation were included in these studies, and surprisingly the human PAC gene was retained in hybrids retaining either of these reciprocal translocation chromosomes. Southern analysis of these DNAs after digestion with a variety of restriction enzymes provided no evidence for the translocation break point occurring within the PAC gene but a possible reduplication of a "small" chromosomal region at the breakpoint could be present; other explanations cannot be excluded. No RFLPs were detected at this locus but a useful SSCP was identified by PCR amplification of a 350 nucleotide sequence from the 3'-untranslated region of the cDNA. This SSCP was used to analyse the 40 CEPH families and linkage analysis allowed ordering of the gene with respect to other loci in this region. Since the gene is likely to be important in growth regulation, this highly informative polymorphism will be very useful in evaluating it as a possible candidate gene in diseases including neoplasms.

Three different members of a multigene family of plasma membrane  $\text{Ca}^{2+}$  ATPases have previously been mapped to human chromosomes 1, 12, and 3 in this laboratory. Recently, we have localized a fourth member (PMCA3) to the human X-chromosome, and it has been localized to bands Xq26-q28 on the distal long arm by analysis of hybrids containing specific breaks involving this chromosome. This is of particular interest because the gene exhibits tissue specific regulation of expression, with particularly high levels of expression in the nervous system. Several diseases involving the nervous system have previously been mapped to this chromosomal region, and PMCA3 can be considered a candidate gene in these diseases. More refined localization of PMCA3 is currently being obtained by in situ hybridization and linkage analysis.

A DNA-damage-inducible gene, GADD153, was recently mapped to chromosome 12q13.1-q13.2 in collaboration with N.J. Holbrook et al. by Southern analysis of hybrid cell DNAs and by in situ hybridization. Since there have been reports of specific chromosomal aberrations within this same region in several different types of neoplasms including liposarcomas, we speculated that the gene might be involved in one of these neoplasms. Other investigators (i.e. F. Mitelman, N. Ron, and their colleagues) explored this possibility, and they found that this gene is consistently rearranged in liposarcomas with a t(12;16) translocation and the rearrangement is presumably related to tumorigenesis. Based upon this fact and the recent success in showing the involvement of IL-2RY in XSCID, we



are currently evaluating several other cloned genes as possible candidates for genetic diseases including the recently described hereditary nonpolyposis colorectal cancer (HNPCC) gene on chromosome 2. This requires cloning genomic sequences for some of these genes and identifying highly informative polymorphisms at these sites; these studies are under way.

Another project has involved identifying, sequencing, and characterizing DNA tracts containing highly polymorphic short tandem repeats, and nine (AAT)<sub>n</sub> and one (AAC)<sub>n</sub> tracts on chromosome 22 have been so analyzed. All these loci contain at least nine repeats of the triplet and they are all multiallelic, usually with about six to eight alleles. Seven of the ten have heterozygosities of at least 0.7 and PIC's of at least 0.6; the remainder have heterozygosities of at least 0.39. No stutter is encountered during PCR amplification of these tracts, and the results are very reproducible and easy to interpret. Unfortunately, one of these tracts occurs within the immunoglobulin  $\lambda$  locus, and somatic deletions occur in B-lymphocytes, resulting in frequent hemizyosity and occasional null alleles in this case. Hence, this locus can only be analyzed in a small fraction of the meioses. The 10 loci are dispersed over nearly the entire length of chromosome 22, spanning about 70 cM. These loci can all be ordered by multipoint linkage analysis, and they provide a framework for ordering a large number of genes on this chromosome. Nine of these ten AAT and AAC repeats are found in the polyA tract of Alu sequences, suggesting a mechanism by which these tracts arose. Alu is not found on the other side of these sequences. Despite the presence of Alu, these sequences are useful and can be used as polymorphic markers. In some cases, a primer has been prepared that anneals within the Alu sequence at a site of maximum divergence from the consensus Alu sequence; in other cases, DNA sequencing has been extended beyond the Alu and a unique sequence primer has been prepared. Readily interpretable results are obtained in both situations. Another polymorphic (AAT)<sub>n</sub> tract was isolated from the chromosome 22 library, and it exhibited no linkage to the other markers. We have mapped it to chromosome 3q, and it is an informative (i.e., PIC greater than 0.7) marker on this chromosome.

#### Publications:

Yoneda K, McBride OW, Korge BP, Kim IG, Steinert PM. The cornified cell envelope: loricrin and transglutaminases, *Jap J Dermatol* 1992;19:761-4.

McBride OW. Gene mapping by genetic linkage and somatic cell hybrid analysis. In: Kirsch IL, ed. *Causes and consequences of chromosomal aberrations*. Boca Raton, FL: CRC Press Inc., 1992;29-48.

Motokura HF, Yi HF, Kronenberg HM, McBride OW, Arnold A. Assignment of the human cyclin D3 gene (CCND3) to chromosome 6p-q13, *Cytogenet Cell Genet* 1992;61:5-7.

Yoneda K, Hohl D, McBride OW, Wang M, Cehrs KU, Idler WW, Steinert PM. The human loricrin gene, *J Biol Chem* 1992;267:18060-6.

Park JS, Luchty JD, Wang MG, Fargnoli J, Fornace AJ, McBride OW, Holbrook NJ. Isolation, characterization and chromosomal localization of the human GADD153 gene, *Gene* 1992;116:259-67.

- Mitchell A, Bale AE, Lee BJ, Hatfield D, Harley H, Rundle SA, Fan YS, Fukushima Y, Shows TB, McBride OW. Regional localization of the selenocysteine tRNA gene (TRSP) on human chromosome 19, *Cytogenet Cell Genet* 1992;61:117-20.
- Compton JG, DiGiovanna JJ, Santucci SK, Kearns KS, Amos CI, Abangan D, Korge BP, McBride OW, Steinert PM, Bale SJ. Epidermolytic Hyperkeratosis completely cosegregates with the type II keratin gene cluster on chromosome 12q, *Nature Genetics* 1992;1:301-5.
- Yamaguchi N, Kimura S, McBride OW, Hori H, Yamada Y, Kanamori T, Yamakoshi H, Nagai Y. Molecular cloning and partial characterization of a novel collagen chain,  $\alpha 1$  (XVI), consisting of repetitive collagenous domains and cysteine containing non-collagenous segments, *J Biochem* 1992;112:856-63.
- Yi H, Donohue SJ, Klein DC, McBride OW. Localization of human hydroxyindole-O-methyltransferase gene to the pseudoautosomal region: implications for mapping of psychiatric disorders, *Hum Molec Genet* 1993;2:127-31.
- Fleischhauer K, McBride OW, DiSanto JP, Ozato K, Yang SY. Cloning and chromosome mapping of human retinoid X receptor B: selective amino acid sequence conservation of a nuclear hormone receptor in mammals, *Hum Genet* 1993;90:505-10.
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- Goldstein AM, Dracopoli NC, Ho EC, Fraser MC, Kearns KS, Bale SJ, McBride OW, Clark Jr WH, Tucker MA. Further evidence for a locus for cutaneous malignant melanoma-dysplastic nevus (CMM/DN) on chromosome 1p, and evidence for genetic heterogeneity, *Am J Hum Genet* 1993;52:537-50.
- Sher T, Yi HF, McBride OW, Gonzalez FJ. cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor, *Biochemistry* 1993;32:5598-604.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 05203-25 LB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Immunochemical Purification and Characterization of Immuncytes and Components		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
M.G. Mage	Immunochemist	LB NCI
L.L. McHugh	Biologist	LB NCI
L. Lee	Visiting Fellow	LB NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Biochemistry, DCBDC		
SECTION Biosynthesis Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2.50	PROFESSIONAL: 2.50	OTHER:
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 <span style="float: right;">B</span> <input type="checkbox"/> (a2) Interviews      AIDS research: 50%		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Our laboratory has a long-standing interest in immunochemical methods for cell separation, and in developing engineered macromolecular reagents for use in studying relatively low affinity interactions (such as those of MHC molecules with peptides and T cell receptors). We currently study engineered recombinant single chain Class I MHC molecules, with respect to their cell biology, immune function, and potential use as vaccine components.</p> <p>During this year, we have further characterized our original construct- the recombinant soluble single chain H-2D<sup>d</sup> Class I MHC molecule, sscH-2D<sup>d</sup>. We have found that sscH-2D<sup>d</sup> as secreted contains a complement of bound endogenous peptides similar to those found in wild type two chain molecules. This provides further evidence that recombinant sscH-2D<sup>d</sup> has a native conformation. We have developed a procedure for affinity purification of the single chain molecules, and for their uniform loading with a single antigenic peptide. Such uniform loading makes it possible to investigate their immunogenicity.</p> <p>Class I molecules function in nature as cell surface molecules. Dr. Lee has engineered a recombinant gene for a cell-surface bound single chain Class I MHC molecule. Stable L cell transfectants with this gene express the single chain molecule on their surface. The cells can bind an antigenic peptide from HIV gp120, and are functional in antigen presentation to T cells. The single chain molecule can also be stably expressed by cells that lack endogenous <math>\beta</math>2-microglobulin. This shows that the single chain molecule uses its own covalently linked <math>\beta</math>2m, rather than endogenous <math>\beta</math>2m.</p> <p>Finally, we have constructed recombinant genes for three additional single chain Class I MHC molecules: H-2D<sup>d</sup> with a covalently linked antigenic peptide, H-2L<sup>d</sup>, and a human Class I MHC molecule: HLA/A2.</p>		

Project DescriptionObjectives:

Our goals are to develop improved methods and macromolecular reagents for studying receptor-ligand interactions, for targeting tumor cells and autoimmune cells, and for immunizing T cells, and to apply them to the development of novel or improved vaccines and therapies for conditions such as AIDS, cancer, and autoimmunity.

Major Findings:

Class I MHC molecules bind peptides from endogenously synthesized proteins and display them on the surface of infected cells as an MHC-peptide complex. This complex is recognized by the specific receptors of T cells. Our recombinant single chain Class I MHC molecules, unlike the wild-type two chain molecules, have a non-dissociable  $\beta 2$  microglobulin component. Further studies of these recombinant molecules have led to several findings:

- A. We have developed an affinity purification procedure for the recombinant soluble murine H-2D<sup>d</sup> single chain molecule. This has allowed us to show that the secreted single chain molecules contain a complement of endogenous peptides similar to those of wild-type two chain molecules. This provides further evidence that the recombinant molecule has a native conformation.
- B. By including a large excess of an antigenic peptide from HIV gp120 in the elution buffer during affinity purification, we have been able to load the single chain molecules uniformly with a single peptide. This makes it possible to study the immunogenicity and potential use of single peptide. This makes it possible to study the immunogenicity and potential use of single chain molecules as vaccine components.
- C. In nature, Class I MHC molecules function as cell surface molecules. Dr. Lee has engineered a recombinant gene for a cell surface membrane-bound single chain Class I molecule, mscH-2D<sup>d</sup>. Stable L cell transfectants express mscH-2D<sup>d</sup> on their surface. The cells can be pulsed with an antigenic peptide from HIV gp120, and can present it to the appropriate T cell hybridoma. This shows that the cell surface mscH-2D<sup>d</sup> folds correctly and is functional.
- D. Cells that lack endogenous  $\beta 2$ -microglobulin can stably express the single chain molecule mscH-2D<sup>d</sup> on their surface. This shows that the single chain molecule uses its own covalently linked  $\beta 2m$ , rather than endogenous  $\beta 2m$ .
- E. The cell surface single chain molecule mscH-2D<sup>d</sup> can also be expressed by cells that are defective for peptide transport into the endoplasmic reticulum. Such cells express "empty" Class I MHC molecules when grown at 27°. By incubating the cells with an excess of antigenic peptide from HIV gp120, the cell surface mscH-2D<sup>d</sup> molecules can be uniformly loaded with this peptide, and are functional in antigen presentation. This makes it possible to study the T cell response to uniformly peptide-loaded cell surface Class I MHC molecules.

F. We have constructed a recombinant gene for single chain H-2L<sup>d</sup>, in order to generalize from our findings with the first single chain molecule, and to attempt co-crystallization with an H-2Ld-specific soluble T cell receptor.

G. We have constructed a recombinant gene for single chain H-2D<sup>d</sup> encoding a covalently attached antigenic peptide from HIV gp120. This type of construct should allow the synthesis of the entire Class I heterotrimer of heavy chain,  $\beta$ 2m, and peptide, as a single covalently linked polypeptide chain. This can greatly simplify the preparation of such molecules for use as vaccine components.

H. To allow the extension of our studies to the human situation, we have also recently constructed a recombinant gene for the most common human Class I molecule, HLA/A2.

Publications:

Mage M, Lee L, Ribaud RK, Corr M, Kozlowski S, McHugh L, Margulies DH. A recombinant, soluble, single chain class I major compatibility complex molecule with biological activity, Proc Natl Acad Sci USA 1992;89:10658-62.

Catipovic B, Dal Porto J, Mage M, Johansen TE, Schneck JP. Major histocompatibility complex conformational epitopes are peptide specific, J Exp Med 1992;176:1611-8.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05231-19 LB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Role of Subunit Interactions in Enzyme Chemistry and Cellular Regulation

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

C. B. Klee	Chief, Protein Biochemistry Section	LB	NCI
A. S. Stump	IRTA Fellow	LB	NCI
I. Myagkikh	Visiting Fellow	LB	NCI
H. Ren	Visiting Associate	LB	NCI
M. Leitner	Visiting Scientist	LB	NCI
X. Wang	IRTA Fellow	LB	NCI

COOPERATING UNITS (if any) Dr. A. Bax, NIDDK; Dr. S. Schreiber, Harvard University; Dr. S. Burakoff, Dana-Farber Cancer Institute, Boston; Dr. Ching Kung, University of Wisconsin; Dr. O. Wesley McBride, NCI; Dr. Eva Mezey, NINDB; Dr. J. Anglister, NIDDK (on leave from the Weizmann Institute, Israel)

## LAB/BRANCH

Laboratory of Biochemistry, DCBDC

## SECTION

Protein Biochemistry Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

6.00

## PROFESSIONAL:

5.50

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

Work in this laboratory is aimed at elucidating the mechanism of stimulus-response coupling mediated by  $Ca^{2+}$  and calmodulin. The calmodulin-stimulated protein phosphatase, calcineurin, is used as a model system. Our ultimate goal is to elucidate the structure of the calcineurin/calmodulin complex in order to understand how calmodulin activates this important enzyme. Our efforts, this year, were focused on the role of the  $Ca^{2+}$ -binding regulatory subunit of calcineurin, calcineurin B, in the regulation of the protein phosphatase activity.  $Ca^{2+}$  binding to calcineurin B was shown to be absolutely required for enzymatic activity as well as for the calmodulin stimulation of the enzyme. Myristoylated and unmyristoylated calcineurin B have been expressed in high yield in *E. coli*. The  $Ca^{2+}$  binding properties of the recombinant proteins are similar to those of native calcineurin B but substitution of the myristoylated calcineurin B of the native enzyme by unmyristoylated calcineurin B yields inactive enzyme. The three-dimensional structure of recombinant calcineurin B has been determined in collaboration with Ad Bax and Jacob Anglister (NIDDK). Recombinant calcineurin B $\beta$  has also been expressed in high yield in *E. coli*. It is being recombined with myristoylated calcineurin B to reconstitute enzymatically active enzyme and to obtain homogenous preparations of calcineurin suitable for crystal structure determination.

## Project Description

### Objectives:

To study the functional roles of protein-protein interactions in the regulation of cellular processes. 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.

### Calmodulin and Calcium Regulation of Cellular Activity

#### A. Interaction of Calmodulin with Target Proteins and Peptides

The mechanism of calmodulin activation of calcineurin involves the displacement of an autoinhibitory domain by binding of the  $\text{Ca}^{2+}$ -calmodulin complex to a calmodulin-binding domain. Although this mechanism applies to most calmodulin-regulated enzymes, different enzymes interact with and are activated by calmodulin differently. One of our goals is to identify the differences that insure the temporal and topological coordination of  $\text{Ca}^{2+}$  regulated cellular processes.

Calcineurin is one the few calmodulin-regulated enzymes under the control of two different  $\text{Ca}^{2+}$ -binding proteins, calmodulin and calcineurin B, an integral subunit of the enzyme. As expected, the  $\text{Ca}^{2+}$  concentration needed to promote calcineurin interaction with calmodulin and stimulation of its phosphatase activity is decreased by increasing concentrations of calmodulin. Conversely, calcineurin should also increase the affinity of calmodulin for  $\text{Ca}^{2+}$ . The affinity of calmodulin for  $\text{Ca}^{2+}$  was tested in the presence of the isolated calmodulin-binding domain of calcineurin. The affinity of the high affinity sites was increased tenfold, as expected, and that of the low affinity sites was increased even more, 100- to 300-fold (Paul Stemmer).

In contrast to other calmodulin-regulated enzymes, calmodulin stimulation of calcineurin exhibited an absolute requirement for  $\text{Ca}^{2+}$  which could not be eliminated by increasing calmodulin concentration. Thus, calcineurin B is not only required for calcineurin activation, as previously reported, but  $\text{Ca}^{2+}$  binding to calcineurin B is also absolutely required for calmodulin stimulation of the enzyme. The highly cooperative,  $\text{Ca}^{2+}$ -dependent binding of calmodulin to calcineurin allows calcineurin activation over very narrow  $\text{Ca}^{2+}$  thresholds. The requirement for  $\text{Ca}^{2+}$  binding to calcineurin B insures the absolute dependence on stimulated levels of intracellular  $\text{Ca}^{2+}$  for calcineurin activity.

#### B. Calcineurin Structure Function Relationships

The two subunits of calcineurin have been expressed in *E. coli* in order to reconstitute homogenous enzyme suitable for crystallization and structure determination. Myristoylation of the amino terminus is a conserved feature of calcineurin B. Myristoylated and unmyristoylated calcineurin B have been expressed in high yield in *E. coli* to test the role of this post-translational modification in subunit-subunit interaction and enzyme activity. Both recombinant proteins can be purified by a single chromatographic step, bind  $\text{Ca}^{2+}$  with high affinity and exchange with the endogenous

regulatory subunit at very slow rates even in the presence of EGTA ( $k=2.4 \times 10^{-6} \text{ sec}^{-1}$ ). Addition of CHAPS, in the presence of EGTA but not  $\text{Ca}^{2+}$ , increased the rate of exchange ten-fold. The protein phosphatase and *p*-nitrophenylphosphatase specific activities of the reconstituted calcineurins were directly proportional to the extent of myristoylation of calcineurin B. Thus, myristoylated calcineurin B must be used for the reconstitution of enzymatically active calcineurin from its subunits expressed in *E. coli* (Ren Hao).

### C. Physiological Roles of Calcineurin in Cellular Signaling

Two new roles for calcineurin in cellular signaling have been identified using its specific inhibitors the immunosuppressant, FK506, and the synthetic peptide corresponding to the autoinhibitory domain of the enzyme. In collaboration with Dr. P. Greengard and his colleagues (Rockefeller University) we showed that the  $\alpha$ -adrenergic stimulation of the  $\text{Na}^+/\text{K}^+$  ATPase of proximal tubules in the kidney is mediated via activation of calcineurin. Calcineurin was also shown, in collaboration with Dr. F. Maxfield (Columbia University), to play a role in neutrophil chemokinesis by mediating the  $\text{Ca}^{2+}$ -induced detachment of neutrophils from adhesive substrates.

#### Publications:

Ikura M, Barbato G, Klee CB, Bax A. Solution structure of calmodulin and its complex with a myosin light chain kinase fragment, *Cell Calcium* 1992;13:391-400.

Aperia A, Ibarra F, Svensson L.-B., Klee CB, Greengard P. Calcineurin mediates  $\alpha$ -adrenergic stimulation of  $\text{Na}^+, \text{K}^+$ -ATPase activity in renal tubule cells, *Proc Natl Acad Sci USA* 1992;89:7394-7.

Guerini D, Montell C, Klee CB. Molecular cloning and characterization of the genes encoding the two subunits of *Drosophila melanogaster* calcineurin, *J Biol Chem* 1992;267:22542-9.

Hendey B, Klee CB, Maxfield FR. Inhibition of neutrophil chemokinesis on vitronectin by inhibitors of calcineurin, *Science* 1992;258:296-9.

Lipp H-P, Wolfer DP, Qin WX, Klee CB, Heizmann CW. CBP-18, a  $\text{Ca}^{2+}$  binding protein in rat brain: tissue distribution and localization, *J Neurochemistry* 1993;60:1639-49.

Anglister J, Grzesiek S, Ren H, Klee CB, Bax A. Isotope-edited multidimensional NMR of calcineurin B in the presence of the non-deuterated detergent CHAPS, *J Biomolec NMR* 1993;3:121-6.

Grzesiek S, Anglister J, Ren H, Bax A.  $^{13}\text{C}$  line narrowing by  $^2\text{H}$  decoupling in  $^2\text{H}/^{13}\text{C}/^{15}\text{N}$  enriched proteins. Application to triple resonance 4DJ connectivity of sequential amides, *J Am Chem Soc*, 1993, in press.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 05244-16 LB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Transposable Elements in the Human Genome

## 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
G. Swergold	Senior Staff Fellow	LB	NCI
J. McMillan	Visiting Fellow	LB	NCI
A. Clements	IRTA	LB	NCI
H. Hohjoh	Visiting Fellow	LB	NCI
K. Liu	Visiting Fellow	LB	NCI

## COOPERATING UNITS (if any)

Thomas Fanning, Armed Forces Institute of Pathology; K. Ozato and K. Becker, Laboratory of Developmental & Molecular Immunity, NICHD

## LAB/BRANCH

Laboratory of Biochemistry, DCBDC

## SECTION

Gene Structure and Regulation Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.85

## PROFESSIONAL:

4.6

## OTHER:

0.25

## 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.)

Work has continued on the transcription and translation of LINE-1 retrotransposons (LHs) in human teratocarcinoma cells and on the characterization of the polypeptides encoded by these elements. The aim is to understand the mechanism and regulation of transposition.

The DNA sequences near the 5' ends of some LHs elements are undermethylated in cell lines synthesizing p40 but are methylated in cells with little or no detectable LHs expression, suggesting that methylation state plays a role in LHs expression.

Translation of LHs ORF2, which is separated from ORF1 by 33 base pairs including several in-frame stop codons, was studied *in vitro*; no ORF1-ORF2 fusion protein was detectable, translation appears to initiate with the first AUG codon in ORF 2, and the absence of ORF1 translation had no effect on ORF2 translation. These results indicated that ORF2 translation is independent of ORF1 translation, *in vitro*. Experiments to study translation in teratocarcinoma cells upon transient transfection with appropriate DNA constructs lead to a similar conclusion; ORF2 translation is initiated internally within the LHs mRNA and is independent of ORF1 translation. Thus, the inhibition of ORF1 translation that results from insertion of a stable stem-loop structure in the 5' UTR, is accompanied by an increase in ORF2 translation.

p40, the translation product of LHs ORF1 and segments of the polypeptide encoded by ORF2 have been expressed in pRSET expression vectors in *E. coli*. Large amounts of p40 have been purified from the bacterial cells under denaturing conditions and renatured by controlled dialysis. Homomultimeric complexes of p40 form and appear to involve both disulfide and hydrophobic bonds. Experiments with p40 missing its carboxyl terminal region but retaining the leucine zipper portion of the molecule indicated that the carboxyl terminus facilitates formation of the complexes.

Objectives:

The LINE-1 element (L1Hs) is the only known transposable element in the human genome; it is a class II (or nonLTR) retrotransposon. At least one such element of the approximately 3500 full length L1Hs elements in the genome, an allele (L1.2B) at the L1RE-1 locus on chromosome 22, appears to be actively transposing. Our aim is to understand the mechanism and the regulation of L1Hs transcription and translation in order to elucidate the mechanism and control of transposition. Previous work demonstrated: 1) that L1Hs transcription and translation is most abundant in human teratocarcinoma cells and tumor cells of germ line origin, of cells tested, 2) that p40, the phosphorylated polypeptide product of the first of the two L1Hs open reading frames (ORF1) is detectable in such cells where it occurs in cytoplasmic multimeric complexes, presumably involving the leucine-zipper motif, and 3) that the first approximately 670 bp of the 900 bp GC-rich 5' UTR contains all the *cis*-acting sequences required to promote transcription in a cell-specific manner.

Major Findings:L1Hs Translation

L1Hs mRNA has a number of features likely to affect the efficiency of translation of both ORF1 and ORF2 (which encodes the reverse transcriptase): 1) a 900 residue long, GC-rich 5' UTR with the potential to form stable secondary structures and at least one AUG and associated short open reading frame (in each putatively active element and cDNA thus far characterized), 2) a 33 base region separating ORF1 and ORF2 (which are in the same frame) and containing multiple, in-frame stop codons. Experiments were carried out to determine if ORF1 translation to p40 is consistent with the scanning ribosome model. A very stable hairpin structure was inserted into a region of the 5' UTR known to be unnecessary for efficient transcription and its effect on translation of ORF1 and ORF2 was measured. Translation of ORF1 and of a *lacZ* reporter gene fused in-frame after the first few ORF1 codons was inhibited both *in vitro* (reticulocyte lysate) and *in vivo* (after transfection into teratocarcinoma cells, NTera2D1). These results are compatible with a model for ORF1 translation initiation in which ribosomes scan from a point 5' of nucleotide 661, the site of the hairpin insertion. In *in vitro* experiments designed to study ORF2 translation, polypeptide products were readily detectable; both ORF2 itself and a *lacZ* reporter gene fused inframe after the first few ORF2 codons was investigated. The size of the largest product indicated that translation initiated at or near the first methionine codon, although this codon is not in an optimal context for initiation. There was no indication that an ORF1-ORF2 fusion protein formed, suggesting that there is no readthrough of the in-frame stop codons in the interORF region. Moreover, the absence of ORF1 translation, caused either by a frameshift and early termination in ORF1 or by the deletion of much or all of ORF1, had no effect on ORF2 translation. Thus, the translation of ORF2, *in vitro*, is independent of the translation of ORF1. The same appears to be true in teratocarcinoma cells as indicated by experiments utilizing the constructs with the stable hairpin structure inserted in the 5' UTR (described above). Although ORF1 translation was inhibited in the presence of the hairpin in transiently transfected NTera2D1 cells, ORF2 translation, which is in any case inefficient, was increased (using a *lacZ* reporter gene). Thus, the level of ORF2 expression was not directly proportional to the level of ORF1 expression. The results suggest that ORF2 is not translated by attached ribosomes reinitiating following termination of ORF1 translation. Rather, the data are compatible with a model whereby translation of ORF2 is independently initiated, internally.

## L1Hs Polypeptides

The size of ORF1 predicts a polypeptide product of 338 amino acids and the size of p40 in teratocarcinoma cells is consistent with the predicted molecular weight. ORF1 has no significant homology to proteins or DNA sequences contained in GenBank, except for the presence of the leucine zipper motif and its function, if any, with respect to L1Hs transposition is unknown. As one strategy to facilitate study of the significance of p40, an efficient bacterial expression system was developed. L1Hs ORF1 from L1.2 was incorporated into a pRSET expression vector containing a 5' (His)<sub>6</sub> affinity tag and a T7 promoter. Using this vector, expression of p40 was abundant in appropriate *E. coli* cells and large amounts of denatured p40 were purified to homogeneity from bacterial extracts with a Ni<sup>2+</sup>-NTA-agarose column (which binds the histidine tag) followed by Sephacryl gel filtration. Upon controlled dialysis to bring the solution to non-denaturing conditions, homomultimeric complexes formed. Both disulfide bonds and hydrophobic interactions appear to be involved in complex formation. p40 lacking the carboxyl terminal 100 amino acids (eliminated by expression and purification of the polypeptide product of a construct lacking the 3' end of the coding region) but retaining the leucine zipper motif was expressed and purified in the same manner. Complex formation also occurred with the shortened p40 although less efficiently, suggesting that the carboxyl terminal region contributes to the stability of the detected complexes. The purified p40 was also used to purify polyclonal antiserum to p40.

ORF2 coding sequences from L1.2A were also cloned into the pRSET expression vector. Extracts from *E. coli* cells expressing the recombinant DNAs were screened for accumulation of ORF-2 derived fusion proteins. Attempts to express the full length of ORF2 in such a system were not successful. Therefore, subfragments of ORF2 were cloned. Four fusion proteins which bind to Ni<sup>2+</sup>-NTA-agarose columns and react with ORF2-specific antisera have been detected. The largest of these contains 56 percent of the predicted full-length ORF2 protein including the region predicted to encode the reverse transcriptase of L1Hs. Attempts to overexpress fusion proteins encoded by the 3' half of ORF2 under the same conditions have not been successful.

## Future Plans

During the next year effort will concentrate on L1Hs proteins. Studies will be directed to understand the intracellular localization of p40 and its multimerization using both cell extracts and the *E. coli* produced protein. Work will continue on the characterization of the ORF2 polypeptide products produced in *E. coli*, including their potential functional properties; renaturation of the denatured, purified proteins will be a major focus. If active reverse transcriptase can be produced, work will concentrate on the mechanism of reverse transcription. The intracellular localization of L1Hs polypeptides and RNA will be investigated to determine if any or all of these molecules occur in particles.

## Publications:

Singer MF, Fanning TG, Leibold DM, Swergold GD, Thayer RE. Moveable elements in the human genome. In: Mongkolsuk S, ed. Biotechnology and environmental science: molecular approaches, New York: Plenum,1992;7-11.

Holmes SE, Singer MF, Swergold GD. Studies on p40, the leucine zipper motif-containing protein encoded by the first open reading frame of an active human LINE-1 transposable element, *J Biol Chem* 1992;267:19765-8.

Goldfarb LG, Petersen RB, Tabaton M, Tabaton M, Brown P, LeBlanc AC, Montagna P, Cortelli P, Julien J, Vital C, Pendelbury WW, Haltia M, Wills PR, Hauw JJ, McKeever PE, Monari L, Schrank B, Swergold GD, Autilio-Gambetti L, Gajdusek DC, Lugaresi E, Gambetti P. Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism, *Science* 1992;258:806-8.

Thayer RE, Singer MF, Fanning TG. Undermethylation of specific LINE-1 sequences in human cells expressing a LINE-1-encoded protein, *Gene* 1993, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 CB 05258-14 LB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Studies of Eukaryotic Gene Regulation		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
B.M. Paterson	Chief, Biochemistry of Gene Expression Section	LB NCI
J. Eldridge	Biochemist	LB NCI
Q. Wei	Visiting Fellow	LB NCI
M. Shirakata	Visiting Fellow	LB NCI
I. Karsch-Mizrachi	Visiting Scientist	LB NCI
D. Hursh	Sr. Staff Fellow	LB NCI
T. Jones	Biologist	LB NCI
J. Zhang	Visiting Fellow	LB NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Biochemistry, DCBDC		
SECTION Biochemistry of Gene Expression Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.53	7.33	0.20
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		B
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  We have continued our studies on the genes involved in cellular commitment of the myoblast and its subsequent differentiation during myogenesis. The precursor myoblast is defined early in development within the somite compartment as a group of cells expressing the MyoD family of gene regulatory factors, MyoD, myogenin, myf5, and MRF4. The expression of these factors not only marks the committed cell but also plays a role in activating the muscle-specific genes during terminal differentiation. We have isolated these four genes in the chicken and have been studying their pattern of expression in development, both in vivo and in vitro, determining the role of phosphorylation in their functional regulation, and identifying other related bHLH proteins that interact with these myogenic factors to regulate their function. In order to take a genetic approach to the study of myogenesis we have also isolated the MyoD homolog in Drosophila where it is a single gene that marks a subset of muscle precursor cells. By studying the Drosophila gene, the proteins that regulate its function, and the upstream and downstream genes in the myogenic pathway in the fly, we hope to identify correlates in the vertebrates to help us understand more completely vertebrate myogenesis.		

## Project Description

### Objectives:

We would like to understand the molecular basis for the control of eukaryotic gene expression during development and cellular differentiation.

### RESEARCH PROJECTS AND MAJOR FINDINGS.

#### **Studies on the avian myogenic regulatory factors.**

##### **1. Structural and functional characterization of the myogenic regulatory gene CMD1**

Using the CMD1 cDNA clone, we isolated the CMD1 gene with the intention to characterize regulatory regions of the CMD1 gene promoter involved in muscle specific activation and in auto activation, as demonstrated in the activation of the endogenous MyoD gene with the expression of the CMD1 protein in 10T1/2 cells. The entire cDNA sequence was contained within an 18kb genomic insert flanked by 8Kb of 5' sequence and 6.5Kb of 3' sequence. A total of 7.5 Kb of this genomic fragment was sequenced. The coding sequence spans approximately 3Kb and is distributed into three exons. The transcriptional start site is located 177 base pairs 5' of the first coding ATG. The minimum muscle specific promoter is contained within the 322 base pairs 5' of the ATG and is functional only in myoblasts and myofibers. This very GC rich sequence with several CpG islands contains a TATA box, but none of the defined muscle specific regulatory elements. Putative MyoD binding sites, or E-boxes, with the core consensus CANNTG, located in a few regions 5' of the minimal promoter, were tested for binding to CMD1 but none proved to be genuine binding sites. This minimal promoter was unable to function in non-muscle cells or in myoblasts grown in BUDR, a thymidine analog that reversibly blocks myogenesis. Non-muscle specific gene promoters were unaffected by the analog. We checked for the ability of the gene itself to convert 10T1/2 cells to muscle. A 9Kb HindIII sub-fragment containing 5 Kb of 5' sequence was capable of converting almost 1% of the 10T1/2 cells to a stable muscle phenotype. Cells transfected with either the CMD1 cDNA in a promoter-less pSV2 vector or in Bluescript KS+ were negative. Interestingly, CMD1 in trans was not able to activate any of its own promoter fragments joined to a CAT reporter construct although the endogenous gene could be activated. These included fragments from -8Kb to -0.32Kb 5' to the initiator ATG. The addition of intron sequences or of 3' sequence elements also did not facilitate auto activation. Additional factors are required for the "auto-activation" to occur.

##### **2. Isolation and characterization of three additional avian myogenic factors: the homologues to the mammalian factors myogenin, myf5, and MRF4.**

A chicken genomic library was screened with the mammalian cDNAs for the myogenin, myf5 and MRF4 genes under conditions of low stringency. The corresponding genomic fragments were then used to screen chicken embryonic and adult muscle cDNA libraries. We obtained the corresponding cDNA clones with complete coding regions. Myogenin was the first clone isolated with an open reading frame of 227 amino acids. In mammals myf5 and MRF4 are linked and this was the case in the avian genome with both genes contained in a 7Kb EcoRI fragment separated by a 2.4Kb spacer and transcribed in the same direction. The avian myf5, cmyf5, has an open reading frame of 258 amino acids and the avian MRF4, cMRF4, 242 amino acids. The three additional avian clones all convert the mouse 10T1/2 embryonic fibroblasts to muscle and behaved like the mammalian clones in DNA binding assays with the E2A bHLH proteins.

Although these clones have been isolated in mammals, avian embryology has been defined in greater detail and subjected to a variety of experimental procedures not attempted in mammals. Therefore a comparison of the two systems was considered worthwhile. In collaboration with Dr. Gary Lyons of

the University of Wisconsin Medical School, Dr. Margaret Buckingham of the Institute Pasteur and Dr. Jean-Claude Perriard of the ETH in Zurich, we compared the embryonic *in situ* expression patterns of MyoD and Myogenin mRNA in the mouse and chicken in relation to the mRNAs for the creatine phosphokinase M (MCK) and B (BCK) isoforms. The former is expressed in muscle whereas the latter is expressed at high levels in embryonic neural tissue. Interestingly, the timing of expression of MyoD and myogenin are reversed in chicken and mouse: in mouse myogenin is expressed 8.5 days p.c. and MyoD appears at 10.5 days p.c. whereas CMD1 is first detected at stage 13 in the chicken and myogenin appears at stage 15. Both factors appear long before MCK is expressed, suggesting the presence of the factors alone is not sufficient to initiate MCK expression. BCK is an excellent early marker for myotomes and neural tissue and is down regulated in muscle when MCK expression begins. In primary cultures of embryonic chick breast muscle the avian MyoD homolog, CMD1, is the major factor expressed before and after differentiation as well.

### 3. Studies on the role of phosphorylation in the regulation of the avian myogenic factor CMD1.

Mouse MyoD was initially described as a nuclear phosphoprotein expressed at similar levels in the nuclei of myoblasts and muscle fibers. However, the muscle structural proteins are only expressed in the muscle fiber and MyoD is thought to play an essential role in the activation of the family of muscle-specific structural genes. The phosphorylation state of MyoD could regulate MyoD function differently in the myoblast and the muscle fiber. We wished to explore this possibility further.

In order to obtain enough phosphorylated CMD1 for our initial studies we turned to the baculovirus system. A variety of proteins from different species have been expressed in insect sf9 cells with the appropriate post translational modifications, including phosphorylation. A great number of technical problems had to be overcome, including routine infection and selection of the sf9 cells expressing only the recombinant virus and the purification of the CMD1 protein. For our initial purification procedure we had to use an antibody affinity column in the final step in order to obtain sufficiently pure material. Our initial yields of material were low due to the insufficient purity of our viral stocks. This problem was eliminated with the use of an entirely new recombinant selection technique.

We constructed a new viral vector containing the human IL-2 receptor gene expressed under a weak early viral promoter. CMD1 protein was under the control of the stronger polyhedron promoter. Sf9 cells expressing the recombinant virus and protein could be purified directly by IL-2 antibody affinity sorting on magnetic beads or, more recently, on goat anti mouse coated tissue culture flasks. This procedure was later combined with the histidine affinity tag in the baculovirus vector and we can now routinely produce and easily purify proteins expressed in the baculovirus system. Using this procedure we were able to obtain enough phosphorylated CMD1 to study its binding properties and some of its biochemical characteristics.

Both the affinity purified CMD1 from muscle cells and the baculovirus produced CMD1 are phosphorylated only on serine. The preliminary phosphopeptide maps are similar for the two proteins with two peptides out of seven showing variation. CMD1 was dephosphorylated with potato acid phosphatase (PAP) then checked for its dimerization and DNA binding properties. Previous work has shown that the myogenic factors will homodimerize weakly but prefer to heterodimerize with the E2a gene products, another set of bHLH proteins involved in the regulation of the immunoglobulin genes that are also expressed in most cells. Binding reactions were performed in the presence or absence of 1mM magnesium with 100mM KCl. In the presence of magnesium the phosphorylated protein cannot homodimerize and therefore does not bind to DNA. However, in the presence of EDTA the phosphorylated protein binds as well as the dephosphorylated CMD1 or as well as the nonphosphorylated protein made in *E. coli*. Heterodimer formation with the avian E2a gene proteins (see below) and DNA binding is only slightly affected with the PAP treated CMD1, by comparison, with the phosphorylated CMD1 binding more efficiently as the heterodimer. These results suggest regulated phosphorylation of CMD1 in the cell could control the available monomer pool and thus

control the concentration of DNA binding complex involved in muscle specific gene activation. We are in the process of determining the phosphorylated site or sites that regulate this function. The role of phosphorylation will also be examined for the other factors and the E2A gene proteins, E12 and E47.

#### 4. Characterization of the avian E2A gene and its encoded proteins

The myogenic factors are thought to bind DNA as heterodimers with E2A-related proteins. This is based upon studies carried out *in vitro* with E. coli proteins or proteins made in cell-free systems, but the actual *in vivo* complex has not been clearly defined. The role of the E2A proteins in myogenesis has not been examined in primary muscle cultures and has only been suggested based upon studies in various mammalian cell lines. We isolated the cDNA clones encoding the entire chicken E12 and E47 homologues and developed reagents to study the role of the E2A proteins in primary avian myogenesis. The E2A proteins are highly conserved between human and chicken. Comparison of amino acid sequence in the various functional domains show an identity of 98% in the bHLH domain, 94% in the A box, 94% in the carboxyl terminus, and 80% in the nuclear localization signal. Similar levels of homology are shared between the E2A proteins and the other E-like proteins such as HEB, E2-2(ITF2) and E2-5(ITF1). Our results to date indicate the E2A proteins are expressed at much higher levels in muscle as compared to liver or brain, as much as ten-fold higher. Immunofluorescent studies demonstrate nuclear concentrations of the E2A proteins increase dramatically with differentiation and this is also reflected with an increase in the mRNAs for these proteins. This is the first demonstration of the regulation of these proteins during myogenesis. Immunoprecipitation studies with whole cell or nuclear extracts from differentiated muscle cells indicate that the E2A proteins are tightly complexed with CMD1, consistent with the idea these proteins are functioning together as a heterodimer.

To look more closely at the role of these E2A proteins in the process of myogenesis, we established an antisense RNA protocol. Both the avian E12 and E47 proteins can trans activate a chloroamphenicol acetyltransferase (CAT) reporter construct with the E2-5 binding sites. Cotransfections with a five-fold molar excess of the antisense expression construct completely block activation by E12 and E47. These same antisense constructs were then cotransfected into primary chick muscle cultures with a beta galactosidase reporter to mark the transfected muscle cells blue. Both the E12 and E47 antisense constructs were able to prevent the differentiation of muscle. This inhibition could be rescued by addition of excess sense construct, ruling out any double stranded RNA effects, and the vector alone had no effect. These results are consistent with the notion that the E2A proteins are involved with the myogenic factors to activate myogenesis. Northern analysis indicates there are three E-protein related transcripts expressed in muscle cells and we are in the process of identifying each transcript. Eventhough total E2A protein increases during myogenesis and the E47/E12 mRNA ratio increases, the absolute total of E2A gene transcript remains relatively constant, suggesting possible transcriptional control for these proteins.

#### Studies on Drosophila myogenesis

One of the difficulties in studying cellular commitment and differentiation during development in vertebrates is the absence of genetics where one can assess the role of a particular gene product with analysis of the mutant gene and the subsequent complementation studies. The process of muscle formation in Drosophila is similar to that in vertebrates in that the mesodermally derived myoblasts withdraw from the cell cycle and fuse to form the multinucleated syncytial muscle fiber. In addition, Drosophila muscle can be culture in mass cultures for detailed analysis. We have isolated the cDNA and the gene for a Drosophila homologue to vertebrate MyoD gene which is called Dmyd for Drosophila myogenic determination factor. This was accomplished using a PCR amplified domain from the bHLH region of CMD1. Remarkably, there is tremendous sequence conservation at both the nucleotide and protein level in this domain between vertebrates and Drosophila. Unlike the vertebrates, however, there is only a single MyoD-like gene in flies. No related genes could be



detected by low stringency Southern blots or PCR analysis with degenerate primers covering the bHLH domain.

The extremely conserved bHLH region suggested Dmyd would also convert mouse 10T1/2 fibroblast to muscle since there were only 6 amino acid changes in this region when compared to vertebrate MyoD and previous studies had demonstrated the bHLH domain itself could induce conversion. Although Dmyd was a highly expressed nuclear antigen in mouse cells, as judged by antibody staining of 10T1/2 cells, it did not activate the myogenic program. Furthermore, Dmyd did not dimerize efficiently with the E2A proteins like the vertebrate myogenic factors. Dmyd made in *E. coli* would bind to the same DNA consensus or E-box as the vertebrate factors, however. This will be discussed in a later section.

In situ studies on mRNA and protein expression during embryonic development revealed Dmyd was a nuclear antigen expressed in a subset of muscle precursor cells organized in segment specific patterns. Furthermore, the antigen is transiently expressed and nuclear levels decrease to within limits of detection just about the time muscle formation begins. Under these circumstances one could not follow convincingly Dmyd expressing cells into newly formed muscle fibers. To circumvent this problem we constructed a transgenic fly that expressed beta galactosidase activity under the regulation of an 8Kb Dmyd promoter fragment. This fly expressed beta-gal activity in a pattern identical to the expression pattern seen with the Dmyd protein in early phases of development. However, unlike the endogenous Dmyd nuclear protein, beta-gal activity could be followed into newly formed muscle, clearly establishing that Dmyd was transiently expressed in a subset of muscle precursor cells, a few of which which were associated with most of the newly formed muscles in the embryo. Experiments with transgenic flies that can express Dmyd under the control of the HSP-70 heat-shock promoter are in progress to see if Dmyd expressed ectopically can alter the muscle pattern or cell fate. A major portion of our research program will be devoted to the study of myogenesis in *Drosophila*. Our first priority is to obtain a deficiency or mutation in the Dmyd gene to determine the mutant phenotype. As far as we know there are no available deficiencies we could find in the stock center or the literature that cover the location of the gene at 95A on the right arm of the third chromosome. We have received a potential candidate from Dr. Paul Schedl of Princeton that extends from 94D to 95A3 but we do not know yet if this removes the gene. An initial P element screen carried out in the laboratory of Ernst Hafen at the ETH in Zurich while I was on sabbatical also produced no mutation in the gene. Apparently the region 95A is rarely a site for the transposition of a P element. We have been able to collect a few P element and Hobo stocks, another class of transposable element, that map in the 95 region and have jumped these out in the hopes we could remove the Dmyd gene. These jump stocks are under analysis. These same stocks will be treated with gamma irradiation to remove the P element in case the jumpouts do not succeed. If we can obtain a deficiency that removes the Dmyd gene we will carry out an EMS mutant screen and look for lethal complementation groups that map over the deficiency. This may get us a point mutation in the gene. If we get a point mutant we would immediately carry out the complementation test with the Dmyd cDNA and all the vertebrate myogenic factors.

Initially only two Dmyd beta-gal transgenic lines were recovered and only one of these lines, the one used in all our studies called 14.1, gave a strong pattern of beta-gal staining. This P element stock, which maps at 47A on the second chromosome, is being mobilized to generate additional insertion sites in order to rule out possible position effects. We are carrying out a more detailed analysis of the Dmyd 8Kb promoter fragment to look for muscle specific enhancer regions and those regions of the promoter that impart the early and late expression patterns for Dmyd.

In the absence of a mutation in the gene we have undertaken another approach to study the role of the Dmyd positive cells in muscle formation. Cahir O.Kane at the University of Warwick in England has developed a cold sensitive Ricin toxin A chain mutation that has been used in transgenic flies to study eye specific enhancer function during development. As long as the flies are maintained at 18 degrees C, the normal temperature for the long term storage of stocks, the toxin is inactive. When the

temperature is raised to 26 degrees C, the normal temperature for the fly, the toxin is active and the cells expressing the toxin are ablated with no effect on adjacent non expressing cells. We have joined the 8Kb Dmyd promoter fragment to the toxin in a P element construct and have two independent lines with the element on the second and third chromosomes, respectively. We have generated balanced stocks of flies in which we hope to eliminate Dmyd expressing cells at various stages of development. Preliminary experiments show the element on the third chromosome is a larval lethal when induced. There is some indication that the Dmyd positive cells in *Drosophila* are equivalent to the muscle pioneer cells in the grasshopper. Pioneer cells are a subset of mesodermal cells that fix to a precise position in the ectoderm, possibly ectodermally determined, and recruit uncommitted mesodermal cells to build muscle. These pioneers establish the muscle pattern prior to building the muscle. If these cells are laser ablated, the remaining mesoderm cannot form muscle. This Dmyd toxin construct should allow us to determine if *Drosophila* uses a similar mechanism. In addition we should be able to use this approach to identify muscle precursor cells at all stages of fly development.

Cells from the four to six hour embryo can be prepared for mass culture and will differentiate into a variety of differentiated cells types including muscle and nerve. If these cells are cultured initially in the presence of EGTA one can select for a population of muscle cells. In this way a variety of mutant or beta gal marked cells can be studied in vitro. We are carrying out such an analysis with the Dmyd beta gal 14.1 line to see if we can identify expressing cells in culture and determine if they act as centers for myoblast fusion.

We would like to know if Dmyd dimerizes with additional proteins. We have tested a variety of *Drosophila* HLH proteins for interaction with Dmyd but with no success. Two approaches to identify potential partner proteins are being used. 32-P labeled Dmyd protein is being used to screen a *Drosophila* T7 expression library, lambda EXlox, prepared from 6-12 hour embryos, the maximum period of Dmyd expression. This library has already been prepared. The second approach involves immuno-trapping of Dmyd-proteinX complexes from extracts of *Drosophila* embryos using magnetic beads coupled with anti-rabbit antibodies. If results are convincing, this procedure could be scaled up to generate reagents to identify the associated proteins.

There is nothing known about the target genes for Dmyd action. We have generated transgenic flies that express Dmyd under the control of the HSP 70 heat shock promoter. One possible approach to identify target genes that has worked for some studies involves expressing the protein of interest in the salivary glands with a heat shock then staining the chromosomes with antibody to the protein to identify potential sites of interaction. Phage or cosmids that include the target site may be available for further analysis.

### Structural studies on the bHLH proteins

Dmyd protein expressed in mouse 10T1/2 fibroblasts, although it is expressed to high levels in nuclei as judged by antibody staining, does not convert these cells to muscle as do the vertebrate myogenic factors. This seemed unusual since the bHLH domain alone can apparently convert cells and there were only 5 amino acid differences in this region between mouse MyoD and Dmyd. The reason for this difference in function was investigated further. We constructed a variety of chimeric MyoD molecules that were expressed both in *E. coli* and transfected into 10T1/2 cells to study this problem in vitro and in vivo. CMD1 containing the Dmyd bHLH domain does not activate an E-box CAT reporter gene efficiently nor does it dimerize with the E2A proteins in normal fashion. This difference is attributable solely to the 5 amino acid changes in the HLH region of Dmyd, all of which are nonhydrophobic in nature. Single amino acid changes and changes involving one helix at a time revealed the importance of these differences in promoting specific dimerization with E2A proteins and activation of an E-box reporter gene.

All members of the bHLH family of proteins have a highly conserved arrangement of hydrophobic amino acids in the helices which are thought to define the hydrophobic interface between the dimerized

monomers. One amino acid, a phenylalanine in helix 1, is essentially invariant but its role in dimerization has not been clearly established. We constructed a graded series of hydrophobic amino acid replacements in this position, increasing in volume and surface area, beginning with alanine then valine, isoleucine, leucine, tyrosine and tryptophan. All the substitutions were detrimental to DNA binding and reporter gene activation except tyrosine, which was almost as efficient as phenylalanine. These two residues are very similar in hydrophobicity, volume and surface area. These experiments place the phenylalanine in helix 1 in a crucial role in the definition of the hydrophobic interface.

The results from these mutational studies lead us to propose a four parallel helix bundle model for the dimer interface with the following characteristics: the loops are to the outside on opposite sides of the dimer, the phenylalanine is buried in the hydrophobic core of the dimer and is interacting with the phenylalanine in the partner molecule as well as with a conserved alanine in helix 2, the crucial nonhydrophobic residues of helix 1 interact with the nonhydrophobic residues of helix 2 on the adjacent monomer stabilizing the dimer through charge interactions, and the longitudinal axis of the dimer is centered over the dyad in the binding site with each basic domain extended as an alpha helix into the major groove. Preliminary results with a back mutation in helix 1 of E12 that should strengthen a predicted charge interaction with helix 2 of the Dmyd HLH domain support this model.

### Studies on the regulation of the beta actin gene

The promoter of the beta actin gene is constitutively expressed in most cell types, including myoblasts, yet the endogenous gene is not expressed in differentiated muscle fibers. This regulation was attributed to a 40 base pair sequence element in the 3' non coding portion of the gene transcript. This element could be inserted into the same 3' region of RSV neo or alpha cardiac actin and the genes would now behave like beta actin when placed in a muscle cell background. Nuclear runon experiments indicated the majority of this regulation was transcriptionally controlled. Since then we have moved this sequence element to various positions within RSV neo in both orientations. The silencer works in the non coding 5' leader, in the coding region, in the 3' non coding region, and down stream of the polyA addition site. The element works poorly 5' to the RSV enhancer. The fact that the silencer works down stream of the poly A addition site supports the nuclear runon data and rules out mRNA stability as a major regulatory mechanism. This regulatory domain was used to screen a lambda gt11 cDNA expression library from differentiated muscle and a potential clone has been isolated. This clone is being analyzed.

Paterson BM, Shirakata M, Nakamura S, Dechesne C, Walldorf U, Eldridge J, Dubendorfer A, Frasch M, Gehring WJ. Isolation and functional comparison of Dmyd, the Drosophila homologue of the vertebrate myogenic determination genes, with CMD1, Great Britain Society for Experimental Biology 1992;89-109.

Hursh DA, Padgett RW, Gelbart WM. Cross regulation of decapentaplegic and ultrabithorax transcription in the embryonic visceral mesoderm of Drosophila, Development 1993;117:1211-22.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 05263-12 LB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## 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	Chief, Developmental Biochemistry Section	LB	NCI
J. Wisniewski	Visiting Fellow	LB	NCI
Michael Kim	Visiting Fellow	LB	NCI
T. Tsukiyama	Visiting Fellow	LB	NCI
A. Orosz	Visiting Fellow	LB	NCI
G. Mizuguchi	Visiting Fellow	LB	NCI
M. Fritsch	IRTA Fellow	LB	NCI
N. Hosakawa	Special Volunteer	LB	NCI
P. Georgel	Special Volunteer	LB	NCI
M. Jedlicka	Special Volunteer	LB	NCI
M. Martinez-Balbas	Special Volunteer	LB	NCI
B. Davis	Laboratory Worker	LB	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Biochemistry, DCBDC

## 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
- (a2) Interviews

B

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

Carl Wu's group has maintained a focus on gene regulation and chromatin structure, with emphasis on the molecular analysis of transcription factors regulating the heat shock protein (hsp) genes. This group has expanded its efforts in the heat stress signal transduction pathway, in order to capitalize on major advances made over the past year, which included the definition of a novel regulatory domain for the heat shock transcription factor HSF, and the demonstration that the cellular environment plays a critical role in modulating the heat shock-dependent trimerization of HSF. The completion of these projects was celebrated by publications in Science and Nature. During this year studies on crucial post-translational modifications of HSF that are induced by heat shock were initiated using mass spectrometry, the most powerful technique currently available for this purpose. Investigations on the role of chromatin structure on heat shock gene expression were also continued by in vitro reconstitution techniques, and preliminary findings on the ability of the GAGA factor to specifically disrupt nucleosomes on the hsp70 promoter were positive. If confirmed by additional studies, this finding may open a new area of research in transcription factor-chromatin interactions with broad significance for gene regulation.

Project DescriptionObjectives:Regulation of heat shock gene expression

We have analyzed nested deletions primarily from the C-terminus of dHSF and hHSF1, and localized a C-terminal regulatory domain for suppression of trimerization. We plan to continue the mutational analysis of dHSF by constructing deletions from the N-terminus and internal portions of the protein, and analyzing the phenotype of the mutant protein after DNA transfection by gel filtration, chemical crosslinking, nondenaturing electrophoresis and DNA binding techniques. We are employing DNA transfection in Drosophila tissue culture cells as a means of assaying mutant HSF proteins, as the expression of dHSF in either bacterial or yeast cells, more facile hosts for genetic manipulation and expression, leads to a loss of regulated trimerization. Our aim is to try to eliminate, to the extent possible, parts of HSF protein which are nonessential for a regulated monomer-trimer transition. We will also utilize the constructs generated above to study HSF transactivation by co-transfection of a reporter gene (CAT) in tissue culture cells.

We are attempting to reconstitute the HSF monomer in vitro by denaturing purified, bacterial or baculovirus overexpressed HSF trimer in GuHCl and renaturation after dilution in a large volume, in order to disfavor formation of trimers (both expression systems produce only trimers). The fidelity of reconstitution will be assessed by a comparison of the proteolytic cleavage profiles and hydrodynamic properties of the natural and reconstituted HSF monomers. As negative controls for renaturation, we will employ the mutant HSF proteins which show constitutive trimerization when expressed in tissue culture cells.

We have initiated a program to determine the post-translational modifications of dHSF using protein purified from normal and heat shock-induced tissue culture cells or Drosophila embryos. We will employ immunoaffinity and conventional chromatography techniques for the purification. Sufficient quantities of high affinity polyclonal and monoclonal antibodies have been prepared for this purpose. HSF peptides will be prepared by chemical or proteolytic cleavage, separated by reverse phase HPLC, and analyzed by microsequencing and mass spectrometry. While there is preliminary evidence for phosphorylation of HSF upon heat shock, we will determine without bias the nature and location of any post-translational modification that may be found in HSF. We expect modifications could occur on both the inert and the induced forms of HSF. We will also determine if similar modifications occur on HSF in response to induction by chemical inducers, and check the modification state of HSF produced from bacterial and baculovirus expression systems, as these proteins will be employed for reconstitution of monomers, and for biophysical and transcriptional studies. To explore enzyme pathways potentially affecting HSF, we will systematically treat tissue culture cells with a wide range of specific enzyme inhibitors and assay for the induction of DNA binding activity and its reversal upon return to nonshock conditions. This study will also identify those pathways which when blocked do not affect HSF induction.

We have been able to over-express and purify milligram amounts of HSF33-163, a 131-residue polypeptide which retains the ability to footprint on the heat shock element (HSE) with an affinity for DNA several hundred- to a thousand-fold lower than the affinity of the intact HSF trimer. We are in the process of characterizing HSF33-163 and its interaction with wild-type and mutant HSEs by analytical ultracentrifugation. We have also been able to prepare HSF33-163 under conditions suitable for structure determination by NMR, and have initiated a collaboration with Dr. Ad Bax (Laboratory of Chemical Physics, NIDDK) for these studies. A determination of the structure of HSF33-163 should reveal a new motif for specific DNA recognition.

In situ studies of protein-DNA interactions from our group and from the laboratories of John Lis and Sarah Elgin have indicated protein binding on the uninduced heat shock promoter at the TATA box and at GAGA sequences located adjacent to the HSEs. The factors interacting with these sequences are likely to be the TATA binding protein (TBP) and the GAGA factor, isolated in the laboratories of Tom Kornberg and Robert Tjian. We are now reconstituting the native chromatin organization of the hsp70 promoter using the Drosophila nucleosome assembly system and purified transcription factors. The Drosophila GAGA clone has been expressed in a baculovirus expression system with an affinity tag and purified by chromatography. Preliminary studies indicate that GAGA factor has the ability of specifically disrupting nucleosomes from the hsp70 promoter. If substantiated, this finding may open a new area of research in transcription factor-chromatin interactions with broad significance for gene regulation.

#### Publications:

- Lis J, Wu C. Heat shock factor. In: Yamamoto KR, McKnight SL, eds. Transcriptional regulation. New York: Cold Spring Harbor Press, 1992; Book 2: 907-30.
- Brown JL, Wu C. Repression of the segmentation gene fushi tarazu by ectopic expression of tramtrack, Development 1993;117:45-58.
- Rabindran SK, Haroun, R, Clos J, Wisniewski J, Wu C. Regulation of heat shock factor trimerization: role of a conserved leucine zipper, Science 1993;259: 230-4.
- Lavorgna G, Karim FD, Thummel CS, Wu C. Potential role for a FTZ-F1 steroid receptor superfamily member in the control of Drosophila metamorphosis, Proc Natl Acad Sci USA 1993;90:3004-8.
- Clos J, Rabindran SK, Wisniewski J, Wu C. Induction temperature of human heat shock factor is reprogrammed in Drosophila cells, Nature 1993, in press.
- Westwood JT, Wu C. Activation of Drosophila heat shock factor: conformational change associated with a monomer to trimer transition, Mol Cell Biol 1993, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201 CB 05264-12 LB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Characterization of IAP Proviruses Expressed in Normal and Transformed B-Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
K. K. Lueders E. L. Kuff	Research Chemist Chief, Biosynthesis Section	LB NCI LB NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Biochemistry, DCBDC		
SECTION Biosynthesis Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 0.4	PROFESSIONAL: 0.4	OTHER:
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.) The LTRs of expressed IAP elements show variations in sequence, and contain at least five binding sites for nuclear factors. Expression requires hypomethylation of the 5' LTR regulatory regions. Thus, expressed IAP elements are an indicator of the methylation status of their location in the genome and also reflect the presence and balance of particular factors. A limited and highly characteristic set of IAP elements (designated LS-elements) is expressed in normal mouse B-cells (Kuff AR 91). Plasmacytomas generally express higher levels of RNA. We characterized the IAP elements expressed in plasmacytoma MPC11 by sequence analysis of 22 cDNA clones. While the LTRs of the tumor cDNAs were all highly related by sequence, none of the clones were of the LS type (Lueders AR 92). The MPC11 LTRs were 5 to 6-fold more active than an LS cDNA LTR when tested for promoter activity after transfection into S194 plasmacytoma cells. We observed that the tumor-derived cDNAs differed from the LS cDNAs in the sequence of an ATF site; the MPC11 LTRs contain a canonical core sequence TGAGGTC A (ATF-PC) while the LS cDNAs contain an altered sequence (ATF-LS). Nuclear extracts from S194 cells reacted at much lower concentrations in gel shift assays with an ATF-PC oligonucleotide than with an ATF-LS probe. The ATF-PC probe detected multiple IAP transcripts in RNA from established plasmacytomas, but gave no reaction with B-lymphocyte RNA. In contrast, ATF-LS detected higher levels of IAP transcripts in lymphocyte than in tumor RNAs. The results indicate that expression of IAP elements in transformed B-cells is selective for a different set of regulatory sequence variants than those expressed in normal B-cells.		

## Project Description

Objective:

To determine the basis for expression of particular IAP elements in normal and transformed B-cells.

Major Findings:

We have characterized expressed IAP elements to determine the basis for selection of particular elements for expression. IAP genes expressed in lymphocytes of BALB/c mouse (lymphocyte specific or LS-elements) have been shown to represent a restricted set of elements that can be distinguished by specific sequences in the LTR. The LTRs of IAP elements expressed in MPC11 (and other plasmacytomas) differed markedly from the LS-type. Differences were found in recognized nuclear factor binding sites such as Enh1, Enh2, ATF/CRE, and TATA. While a canonical ATF core sequence, TGACGTCA, was present in all of the plasmacytoma IAP clones, the sequence TGAACGTCA was found in every LS LTR. In addition, six other single nucleotide differences were present in sequences flanking the core ATF site.

LTRs from each of two plasmacytoma IAP classes (PC elements) defined by differences in the Enh2 domain of the U3 region were cloned upstream of the chloramphenicol acetyl transferase (CAT) gene and tested for their ability to promote expression after transfection into plasmacytoma cells. Both PC LTRs promoted CAT activity to a similar extent, suggesting that the Enh2 domain did not have a major determining effect on the IAP promoter activity in these cells. The plasmacytoma LTRs were 5 to 6-fold more effective than the LS LTR in promoting CAT activity in plasmacytoma cells.

In contrast, the difference in sequence of the ATF/CRE site between PC and LS cDNAs did correlate with the relative activities on transfection into S194 plasmacytoma cells. RNAs from two plasmacytomas and from normal B-lymphocytes were hybridized with 34-nucleotide probes specific for both the ATF binding site from the plasmacytoma cDNAs (ATF-PC) or the site from the B-lymphocyte cDNAs (ATF-LS). The ATF-PC probe detected high levels of IAP transcripts in plasmacytomas, but gave no reaction with B-lymphocytes. Hybridization with ATF-LS probe detected IAP transcripts from both sources.

The ATF probes were tested in a gel shift assay using extracts of plasmacytoma cells. ATF-PC reacted with a large protein or protein complex over a range of nuclear extract concentrations that gave no reaction with ATF-LS. The results suggest that the preferential expression of PC vs LS type elements in plasmacytoma cells may be determined, at least in part, by the prevalence of factors that bind to the ATF-PC site.

The R-regions of expressed IAP clones show multiple sequence variations. The cloned LTRs from normal lymphocytes could be subclassified on the basis of three characteristic single nucleotide differences in a nine nucleotide motif upstream of the poly(A) signal. The MPC11 cDNA clones exhibited eight related variations in sequence in this region, none of which corresponded to the LS motifs. We



have used probes representing R-region variants and the presence of a conserved methylation sensitive HaeII site in the 5'LTR, to determine the methylation status of individual IAP proviruses. Examination of DNA from three established plasmacytomas has revealed multiple hypomethylated elements, many of them present in all three tumors. These data lead to the important conclusion that hypomethylation of IAP elements does not occur entirely randomly. However, not all IAP elements are demethylated even when their LTR sequences are very similar. It seems likely that the methylation state of many IAP elements is determined by their position in the genome. Mouse plasma cell tumors may have a common pattern of genomic hypomethylation which is reported by the associated IAP elements. The use of subset specific probes provides a means of examining hypomethylation of multiple IAP loci to determine whether distinctive patterns of hypomethylation are present in primary plasmacytomas and other transformed cells.

Publications:

None

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

PROJECT NUMBER  
 Z01 CB 05265-11 LB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

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	Research Chemist	LB	NCI
N. D. Vu	Chemist	LB	NCI
F. Chen	Visiting Associate	LB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Protein Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

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.)

Rat pheochromocytoma PC12 cells and bovine adrenal chromaffin cells are used to study the mechanism of secretion and its regulation by  $Ca^{2+}$  and GTP-binding proteins. Our goals are to determine how  $Ca^{2+}$  induces secretion and to identify some of the proteins involved in the final steps of the secretory process.

Pertussis toxin-modification of either bovine chromaffin cells or PC12 cells results in both a decrease in cytoskeletal F-actin and an increase in secretory activity. These effects of pertussis toxin appear to be independent of changes in second messengers. We have found that pertussis toxin-modification of unstimulated PC12 cells results in about a 50% inhibition of protein phosphatase 2A, a major cellular serine/threonine-specific protein phosphatase. This decrease in phosphatase activity appears to occur in the absence of any extracellular agonist and could account for both the increase in secretory activity and the decrease in cytoskeletal F-actin. Because phosphorylation regulates the activities of many cell surface receptors and/or their intracellular targets, a pertussis toxin-induced decrease in protein phosphatase 2A activity could alter signaling pathways in which G-proteins are not directly involved.

Secretion of norepinephrine by digitonin-permeabilized PC12 cells can be stimulated by the addition of GTPYS in the absence of  $Ca^{2+}$ . GTPYS also stimulates  $Ca^{2+}$ -independent secretion in a number of other types of cells. As in PC12 cells, these stimulations appear to be independent of any known second messenger. The class of GTP-binding proteins responsible for these stimulations has been referred to as  $G_e$ , for exocytosis. Thus far, no  $G_e$  has been isolated. We have isolated from bovine brain membranes a mixture of low molecular weight GTP binding proteins which increases GTPYS-stimulated secretion by digitonin-permeabilized PC12 cells.

We are currently working to identify which low molecular GTP-binding protein is responsible for this stimulation.

### Project description

**Regulation of secretion:** Secretion of neurotransmitters and hormones is usually triggered by an increase in cytoplasmic calcium. The mechanism(s) by which this increase in calcium induces secretion is unknown. The proteins and other molecules involved in both docking and fusion of the secretory vesicles with the plasma membrane are just now being identified, and the mechanism of this fusion is unknown. Our goals are to determine how  $\text{Ca}^{2+}$  induces secretion and to identify some of the proteins involved in the final steps of the secretory process.

To study the mechanism of secretion and its regulation by  $\text{Ca}^{2+}$  and GTP-binding proteins, we use both primary cultures of bovine adrenal chromaffin cells and PC12 cells, an established cell line isolated from a rat pheochromocytoma. When cultured in the absence of nerve growth factor, PC12 cells morphologically resemble chromaffin cells, but when cultured in the presence of nerve growth factor, they resemble sympathetic neurons. Stimulation of both bovine chromaffin cells and PC12 cells with nicotine or  $\text{K}^{+}$ -depolarization results in the  $\text{Ca}^{2+}$ -dependent release of catecholamines. Much of our work is performed with digitonin-permeabilized cells. Treatment of chromaffin and PC12 cells with low concentrations of digitonin permeabilizes the plasma membrane but leaves the secretory vesicles intact. The release of catecholamines by these permeabilized cells is both ATP- and  $\text{Ca}^{2+}$ -dependent and occurs by exocytosis, fusion of the secretory vesicles with the plasma membrane. Permeabilization of the plasma membrane with digitonin allows one to control  $\text{Ca}^{2+}$  and nucleotide concentrations and to introduce proteins into the cell. We use these permeabilized cells to investigate the roles of  $\text{Ca}^{2+}$ , ATP, GTP, the cytoskeleton, and protein phosphorylation in secretion.

### Major findings:

**Inhibition of protein phosphatase 2A by pertussis toxin:** A number of cell surface receptors are linked to their intracellular effector systems through heterotrimeric GTP-binding proteins, G-proteins. Pertussis toxin ADP-ribosylates the  $\alpha$ -subunits of the  $\text{G}_i$  and  $\text{G}_o$  subclasses of G-proteins. This ribosylation uncouples the G-protein from its receptor such that the signaling of the activated receptor to its effector protein is inhibited. Disruption of a signaling pathway by pertussis toxin is frequently used as evidence that a G-protein is involved in a particular signaling pathway.

We have found that pertussis toxin-modification of PC12 cells results in both a decrease in cytoskeletal F-actin and an increase in secretory activity. The secretory activity of these cells can also be enhanced by the activation of protein kinases or by the addition of protein phosphatase inhibitors. Since pertussis toxin-modification of PC12 cells doesn't appear to increase either intracellular  $\text{Ca}^{2+}$  or cAMP levels or activate protein kinase C, we examined the possibility that pertussis toxin-modification might decrease protein phosphatase activity. We have found that pertussis toxin-modification of unstimulated PC12 cells results in about a 50% inhibition of protein phosphatase 2A, a major cellular serine/threonine-specific protein phosphatase. This decrease in phosphatase activity appears to occur in the absence of any extracellular agonist and could account for both the increase in secretory activity and the decrease in cytoskeletal F-actin. Phosphorylation regulates the activities of many enzymes, cell surface receptors, and/or their intracellular targets, and a decrease in protein phosphatase activity could increase their levels of phosphorylation. Thus, a pertussis toxin-induced decrease in protein phosphatase 2A activity could alter signaling pathways in which G-proteins are not directly involved.

**Stimulation of secretion by GTP $\gamma$ S:** Secretion of norepinephrine by digitonin-permeabilized PC12 cells can be stimulated by the addition of  $\text{Ca}^{2+}$  or GTP $\gamma$ S but not GTP. While secretion in the presence of saturating  $\text{Ca}^{2+}$  is not affected by GTP $\gamma$ S, secretion in the absence of  $\text{Ca}^{2+}$  is stimulated 2 to 3 fold by the addition of GTP $\gamma$ S. This stimulation by GTP $\gamma$ S does not appear to result from  $\text{Ca}^{2+}$  release,

activation of protein kinase C, or stimulation of phospholipase A<sub>2</sub>. Cyclic AMP and cyclic GMP have no effect on either basal or GTPγS-stimulated norepinephrine release, and cholera and pertussis toxin have little or no effect on GTPγS-stimulated norepinephrine secretion.

GTPγS stimulates Ca<sup>2+</sup>-independent secretion in a number of other types of cells. As in PC12 cells, these stimulations appear to be independent of any known second messenger. The class of GTP-binding proteins responsible for these stimulations has been referred to as G<sub>ep</sub> for exocytosis. Thus far, no G<sub>ep</sub> has been isolated. We are attempting to isolate a G<sub>ep</sub> from bovine brain membranes. An extract of bovine brain membranes was chromatographed on a sizing column, and the various fractions assayed for their ability to increase GTPγS-stimulated secretion in permeabilized PC12 cells. Fractions enriched in low-molecular-weight GTP-binding proteins gave much larger increases in GTPγS-stimulated secretion than did fractions enriched in heterotrimeric G-proteins. Fractions that increase GTPγS-stimulated secretion have no effect on either basal or Ca<sup>2+</sup>-stimulated secretion. Fractions enriched in low molecular weight GTP binding proteins were further fractionated, and we have obtained a fraction that contains only low molecular weight GTP binding proteins which increases GTPγS-stimulated secretion. We are currently working to identify which low molecular GTP-binding protein is responsible for this stimulation.

#### Publications:

Wu YN, Vu ND, Wagner PD. Anti-14-3-3 protein antibody inhibits stimulation of secretion by chromaffin cell cytosolic proteins. *Biochem J* 1992; 285:697-700.

Wagner PD, Vu ND, Wu, YN. PC12 cells as a model for neuronal secretion. In: DasGupta BR, ed. *Botulinum and tetanus neurotoxins: neurotransmission and biomedical aspects*. New York, NY: Plenum, 1993;105-5.

Rhoads AR, Pauri R, Vu ND, Cadogan R, Wagner PD. ATP-induced secretion in PC12 cells and photoaffinity labeling of receptors. *J Neurochem* 1993, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05267-09 LB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## 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 Biochem. Section, LB NCI  
 D. Chattoraj Microbiologist LB NCI  
 G. Mukhopadhyay Visiting Fellow LB NCI  
 M. Lobočka Visiting Fellow LB NCI  
 H. Lehnherr Visiting Fellow LB NCI  
 J. Dibbens Visiting Fellow LB NCI

## COOPERATING UNITS (if any)

Dr. Martine Couturier, Free University of Brussels; Dr. Marc S. Lewis, BEIP,  
 NCR, NIH

## LAB/BRANCH

Laboratory of Biochemistry

## SECTION

Microbial Genetics and Biochemistry Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

6

## PROFESSIONAL:

6

## 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 continue to study (1) replication control, (2) partition and (3) plasmid addiction of the low copy number plasmid prophage P1 and their contribution to its remarkable stability. (1) Studies of strand opening at the origin of the P1 replicon established that the bacterial initiator, DnaA, in relieving local superhelical tension, permits the two strands to assume very different structures. The difference may be relevant to the unidirectionality of replication. Studies of how *E. coli* chaperone proteins are involved in plasmid replication were undertaken with mutants of the plasmid initiator, RepA, selected for chaperone independence. The evidence suggests that the pairing of DNA-bound RepA monomers is decreased in the mutants, resulting in a copy number increase, in strong support of our previous proposal that the pairing of DNA-bound monomers controls copy number. Apparently the pairing domain of RepA is critical for both the proper folding of that protein and its interactions with chaperones. (2) The P1-encoded system that normally assures plasmid partitioning and consequent stabilization was found to cause plasmid destabilization in a number of situations. Critical variables were shown to be the context of the centromere analog parS and the concentration of the ParB protein, variables expected to influence the fraction of parS bound to ParB. We propose that a context that does not lead to appropriate cyclic variations in the affinity of parS for ParB may lead to destabilization rather than stabilization. Mutations in parS, parB and in vector sequences adjacent to parS, each of which suppresses destabilization, have been characterized. (3) Genes of P1 have been identified that encode an antidote/poison protein pair capable of inducing severe withdrawal symptoms in cells that lose P1. Features of this addiction system that are responsible for an excess of antidote over poison during plasmid retention have been elucidated. Genetic evidence has been obtained that the reversal of this ratio following plasmid loss is due to the selective degradation of antidote by ClpXP protease.

Project DescriptionObjectives:

We seek to understand mechanisms that enable an autonomous genetic element to be stably inherited.

ReplicationA. Strand bias in  $KMnO_4$  reactivity of Pl origin

In the previous annual report we noted that the presence of the host initiator, DnaA, makes the supercoiled Pl origin reactive to the single strand specific reagent  $KMnO_4$ . This result implies that the role of DnaA in Pl plasmid replication is in opening the strands of the origin, as it is in bacterial replication. Origin opening allows loading of the helicase DnaB. Unexpectedly, the  $KMnO_4$  reactivity was restricted to only one strand of the origin. A similar pattern of  $KMnO_4$  reactivity was seen in the absence of DnaA when the linear form of the origin DNA was used instead of supercoiled DNA. This remarkable strand bias can be explained if the bases in the unreactive strand remain stacked (hence resistant to  $KMnO_4$ ) even after duplex opening. The differential reactivity of the DNA strands to  $KMnO_4$  indicates that the two strands of the Pl origin can assume very different structures, but are normally prevented from doing so unless the local superhelical tension is relieved, as by DnaA.

The opening of the Pl origin in the presence of DnaA has been confirmed in studies using a second single strand specific reagent, mung bean nuclease. With this reagent both strands showed nearly equal reactivity. The presumed base stacking differences between the two strands are unlikely to affect this enzyme, as this nuclease attacks the DNA backbone.

It is possible that the differential reactivity of the two strands reflects the fact that the structure of only one of the strands is suitable for loading of DnaB helicase, a feature consistent with the unidirectional mode of replication of the plasmid. The stereo-chemical basis of the differential reactivity remains to be understood.

B. Footprinting analysis of Pl plasmid replication initiator complexes

We showed previously that RepA, the replication initiator protein of plasmid Pl, contacts bases in two consecutive major grooves on the same face of DNA. The evidence was based on the status of G contacts in nucleoprotein complexes as revealed with the methylating agent DMS. In order to know whether bases other than G also contact RepA, depurination and depyrimidation interference experiments were also performed. We additionally identified the DNA phosphate groups that upon ethylation interfered with RepA binding. These results confirmed that RepA binds to one face of DNA and further indicated that there are bases that do not contact RepA directly, but contribute to base and backbone contacts by maintaining the proper structure of the binding site.

C. Replication control by initiator protein-mediated pairing of origin DNAs:  
Role of chaperone proteins

We showed previously that the *E. coli* chaperone proteins DnaJ, DnaK and GrpE promote binding of RepA to the origin, most likely by facilitating protein folding into the active form. The DNA-bound initiators have a second activity. They can pair origins and this pairing is believed to control initiation by steric hindrance to origin function. The role of the chaperone proteins in the pairing process was not known. We have in our collection several RepA mutants that do not require the chaperones for DNA binding. Unexpectedly, the majority of these mutants conferred increased copy number, indicating that they might be defective in initiation control. Three of the mutants have been characterized in vitro for DNA binding and DNA pairing. We find that they are increased in the on-rate of initiator-DNA complex formation, but unchanged in the rate of their dissociation. The mutants were also shown to be defective in the DNA pairing activity. The latter result directly links DNA pairing to initiation control. It also suggests that single amino acid changes which affect the pairing domain cause the on rates to increase, either by allowing more initiator folding into the active form (without the requirement for the chaperones) or by altering the final form of the initiators to increase  $k_{on}$ . Most likely, the DNA binding domain is not altered since the dissociation rates of the mutant initiator-DNA complexes were unchanged.

To explain the frequent occurrence of the control-defective phenotype among the chaperone-independent DNA binding mutants, we suggest that the initiator domain involved in DNA pairing is the target of the chaperones and that the same domain is also used for dimerization of free monomers. In the case of the wild type initiator, the chaperones protect monomers from dimerization and thereby increase their probability to fold into the active form. Apparently, the amino acid changes in the mutants reduced dimerization sufficiently to obviate the need for the chaperones. The changed amino acids may also play a more active role in the pairing process.

In order to address whether the chaperones play any direct role in the pairing process, we studied the role of the chaperones on the functioning of the control locus *incA*. This locus reduces P1 plasmid copy number about eight-fold and is also believed to function by pairing with the origin. The reduction in copy number due to *incA* was similar whether or not the chaperones were present. The chaperones, therefore, do not seem to play any role in replication control. These results suggest that if the pairing domain is the target of the chaperones, they cannot recognize it in the bound form of the initiators. The chaperones most likely recognize the nascent or misfolded forms of the initiator, and not the active species bound to DNA.

Partition

A. Placement of par genes under independently controlled promoters

In order to study the physiology of plasmid partitioning, it is necessary to be able to switch the relevant genes on and off and to quantitate the consequences for plasmid stability within a brief time interval. To this end we have made stable chromosomal insertions of parA and parB under the control of

independently controlled inducible promoters and placed parS in various vectors whose copy numbers can be changed by shifts in temperature or by the use of alternative host bacteria. In addition, the context of parS has been altered so as to isolate it from or subject it to the influence of transcription. This effort has provided the means to switch conveniently between Par function and dysfunction.

#### B. Replicon-independence of Pl par-mediated plasmid destabilization

We previously found that the thermosensitive vector pGB2ts carrying parS is actively destabilized in cells that provide Pl ParB protein at or below levels that can complement a ParB deficiency in partitionable derivatives of Pl. This striking and unexpected behavior is altered by factors that change DNA topology such as transcription and plasmid size and is replicon-independent. By appropriately altering the context of parS we have generated extreme ParB-sensitivity of a parS-carrying plasmid that is replicated from a Pl plasmid origin. Moreover, in the presence of Pl ParA protein a switch between plasmid destabilization and plasmid stabilization can be achieved by a relatively small change in the rate of synthesis of ParB. These results suggest that parS may be poised to participate in a process that can lead to destabilization if circumstances do not permit the parS-ParB interaction to cycle appropriately.

#### C. Characterization of destabilization-resistant parS mutations and non-destabilizing parB mutations

Mutations in parS that confer reduced sensitivity to ParB-mediated destabilization of the plasmid pGB2ts-parB were shown to be located at various ParB binding sites. Changes in only two adjacent base pairs within one of these six putative sites within parS completely eliminated sensitivity to ParB. Mutations in parS that were selected in this way, when present in a context in which active partition could be assayed, were found to confer a parallel deficit in active partition. We take this finding as evidence that routes to stabilization and destabilization branch from a shared point of departure.

We isolated and characterized parB mutations that we selected as expressing ParB protein that is no longer capable of plasmid destabilization. Sequence analysis has revealed that they are located in various regions of parB and predominantly at codons that determine amino acids conserved in homologous proteins of P7, pSLT and F plasmids. These regions may be critical for ParB binding to parS.

#### Addiction

##### A. Identification and characterization of Pl addiction genes phd and doc

We have previously reported evidence that Pl is an addicting plasmid in the sense that its loss causes severe withdrawal symptoms, namely death of the former hosts. We have identified and sequenced the two genes responsible for Pl addiction: phd, a gene that prevents host death, and doc, a gene that causes death on curing. They form a compact operon. Translation of Doc appears to be coupled to that of the upstream Phd region and is probably further disadvantaged relative to Phd translation by the presence of an extraordinary high frequency of rare codons. These mechanisms presumably assure that the antidote is in



excess in cells that retain the plasmid. Preliminary evidence for addiction modules homologous to that of Pl in plasmids belonging to other incompatibility groups has been obtained by Southern blot analysis in collaboration with Dr. Martine Couturier.

B. Involvement of a specific bacterial ATP-dependent protease in plasmid addiction

Bacterial protease mutants were screened to determine whether one or more of them might be required for expression of the addiction phenotype, on the assumption that addiction depends on the rapid loss of antidote (Phd) activity. Of the mutants tested, a clpP and a clpX mutant were found to completely suppress the addiction phenotype. A direct test of the lability of Phd to degradation by the newly recognized ClpXP protease is planned. The protein is being purified and collaborative studies of its proteolysis with Drs. Susan Gottesman and Michael Maurizi are planned.

Publications:

Yarmolinsky MB, Lobočka MB. Bacteriophage Pl. In: O'Brien SJ, ed. Locus maps of complex genomes. 6th ed. New York: Cold Spring Harbor Laboratory Press, 1993; 1.50-61.

Sozhamannan S, Chatteraj DK. Heat shock proteins DnaJ, DnaK and GrpE stimulate Pl plasmid replication by promoting initiator binding to the origin, J Bacteriol 1993;175:3546-55.

Mukhopadhyay G, Chatteraj DK. Conformation of the origin of Pl plasmid replication: initiator protein induced wrapping and intrinsic unstacking, J Mol Biol 1993;231:19-28.

DasGupta S, Mukhopadhyay G, Papp PP, Lewis MS, Chatteraj DK. Activation of DNA binding by the monomeric form of the Pl replication initiator RepA by heat shock proteins DnaJ and DnaK, J Mol Biol, in press.

Papp PP, Chatteraj DK, Schneider TD. Information analysis of sequences that bind the replication initiator RepA, J Mol Biol, in press.

Lehnherr H, Maguin E, Jafri S, Yarmolinsky MB. Plasmid addiction: genes doc and phd of bacteriophage Pl that respectively cause death on curing of prophage and prevent host death when prophage is retained, J Mol Biol, in press.

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

PROJECT NUMBER

Z01 CB 05268-06 LB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Mechanisms of Meiotic Recombination

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

M. Lichten	Senior Staff Fellow	LB	NCI
T-C. Wu	Chemist	LB	NCI
J. Liu	Visiting Fellow	LB	NCI
A. Goldman	Visiting Fellow	LB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Developmental Biochemistry and Genetics

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

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 continued research on the molecular mechanism of meiotic recombination in the yeast Saccharomyces cerevisiae. Our work is focused on two aspects of meiotic recombination. We have examined an initiation event in meiotic recombination, the formation of double-strand breaks in DNA, with the aim of determining the enzyme(s) that form these breaks and the factors that control the frequency and location of breaks. In addition, we have examined the relationship between meiotic recombination and meiotic chromosome pairing, with the ultimate aim of isolating and characterizing DNA intermediates in meiotic recombination and chromosome pairing.

## Project Description

### Objectives:

Our aim is to understand the molecular mechanism of meiotic recombination and chromosome pairing, using the yeast *Saccharomyces cerevisiae* as a model system. We intend to describe at the molecular level the entire process of meiotic recombination, from initial lesions, through intermediate structures, to formation of mature recombinant products. We also intend to examine, again at the molecular level, the changes in chromosome structure (including pairing of homologous chromosomes) that occur during meiosis.

### Major Techniques Employed and Major Findings:

#### A. Chromatin structure determines the location of double-strand breaks.

We have previously shown that formation of double strand DNA breaks (DSB) does not simply involve recognition of a nucleotide sequence, and suggested that chromatin structure played an important role in determining what sites were cut during meiosis. To test this suggestion, we (Michael Lichten and Carol Wu) examined the distribution of DSB sites and of DNase I hypersensitive sites in chromatin in a number of regions of the yeast genome. DSB occurred at many sites in the regions examined, and the distribution of DSB in a region closely parallels the distribution of meiotic crossovers. All the DSB sites identified also displayed elevated levels of cleavage during DNase I digestion of chromatin, and all DNase I hypersensitive sites identified were also DSB sites. The correlation between the distribution of meiotic crossovers and DSB, coupled with the close correspondence we observe between DSB and hypersensitive sites, lead us to suggest that the distribution of meiotic exchange events in the yeast genome is determined, in part, by aspects of chromatin structure. Genes that are active during meiosis, and are thus present in an open chromatin conformation, will recombine more frequently than genes that are repressed. Carol Wu is currently testing this suggestion by examining DSB and meiotic recombination at the *PHO5* locus. Activation of expression of this gene is accomplished by the removal of nucleosomes from its promoter. Preliminary experiments indicate that when the *PHO5* promoter is in this open chromatin conformation, it is frequently the site of DSB during meiosis; when the promoter is repressed and in a closed chromatin configuration, DSB formation is also repressed.

The observation that DNaseI digestion of chromatin closely replicates the pattern of cleavage seen *in vivo* during meiosis raises the possibility that meiosis-induced DSB are formed by a similar activity. Jianhua Liu has recently initiated a study that examines meiotic nuclear extracts for the presence of meiosis-specific endonuclease activities, with the aim of isolating and characterizing the activity responsible for forming DSB.

#### B. Factors that control the level of DSB and recombination at a site.

Carol Wu has shown that the presence of high-level DSB sites suppresses DSB at nearby sites which, by virtue of their chromatin structure, would normally be cut during meiosis. Deletion of a high-level DSB site leads to the restoration of breaks in neighboring sites without affecting their chromatin structure. These effects can be transmitted over considerable distances; they are typically observed over distances of several thousand nucleotides, and can act over distances as large as 20 kb. We believe that these results indicate that enzymes that form DSB during meiosis do not diffuse freely, but rather are distributed among chromosomes in a manner that renders them locally limited.

Remarkably, this suppression is seen not only in *cis*, but also in *trans*. The presence of a high-level DSB site represses breaks at neighboring sites not only on the same chromosome, but also on homologous chromosomes that do not contain the high-level DSB site. This *trans* effect occurs in mutants that block immediately after DSB formation, indicating that homologous chromosomes are already paired at the time of initiation of recombination.

A second set of observations, derived from our studies of meiotic gene conversion between dispersed sets of homologous sequences (ectopic recombination) is also consistent with this view. Ectopic recombination between sequences located on nonhomologous chromosomes (which do not pair during meiosis) occurred 5 to 20 times less frequently than did ectopic recombination between sequences located at different places on homologous chromosomes. This result implies that at least one round of meiotic recombination occurs in the context of already paired chromosomes.

We are currently taking advantage of this effect to search for chromosome elements that are directly responsible for homolog pairing. In addition, we plan to use intra- and interstrand DNA crosslinking reagents, combined with 2-dimensional gel electrophoresis, to obtain direct physical evidence for an early association between homologous chromosomes.

### **Publications:**

Goyon C, Lichten M. Timing of molecular events in meiosis in *Saccharomyces cerevisiae*: stable heteroduplex DNA is formed late in meiotic prophase, *Mol Cell Biol* 1993;13:373-82.

Rocco V, Daly MJ, Matre V, Lichten M, Nicolas A. Identification of two divergently transcribed genes centromere-proximal to the *ARG4* locus on chromosome VIII of *Saccharomyces cerevisiae*, *Yeast* 1993, in press.

Wu TC, Lichten M. Position effects in meiotic recombination. In: Cooper GM, Haseltine FP, Heyner S, Straus JF, eds. *Meiosis II: contemporary approaches to the study of meiosis*. Washington, DC: American Association for the Advancement of Science, 1993, in press.

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

PROJECT NUMBER

Z01 CB 05271-02 LB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Gene Regulation and Function: The bZIP Proteins

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

C. Vinson	Senior Staff Fellow	LB	NCI
D. Krylov	Visiting Fellow	LB	NCI
M. Olive	Visiting Fellow	LB	NCI
S. Boyd	Biologist	LB	NCI

COOPERATING UNITS (if any)

Kelly Thompson and Ernesto Friere, Johns Hopkins University; Ken Zaret, Brown University; Mark Lewis, NIH

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Microbial Genetics and Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

2.6

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

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

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

The last several years have seen the identification of a large number of sequence-specific DNA binding proteins that are important for gene regulation. These proteins use a small number of DNA binding motifs to interact with DNA. This laboratory is focusing on two related motifs that interact with DNA in a sequence-specific manner, the bZIP and the bHLH-Zip motifs. Both motifs bind as dimers to abutted dyad symmetric DNA sequences.

The laboratory is focusing on the dimerization specificity of the bZIP motif, a region termed the leucine zipper. Structural studies indicate that the leucine zipper is a new name for a structure proposed 40 years ago, the coiled-coil. Our structural modeling and experimental results suggest that any bZIP protein could potentially interact with any other bZIP protein to form a dimer bound to DNA. The question is to understand the structural determinants that determine dimerization specificity. Using simple rules of interhelical salt bridge formation, we have predicted novel interactions among known mammalian bZIP proteins. Using the same rules we have designed bZIP proteins that interact with the protein C/EBP better than C/EBP interacts with itself. This engineering of "Dominant-Negative" molecules will be used in a genetic type fashion to help unravel the biological function of C/EBP.

## Project Description

### Objectives:

To understand the function of the bZIP proteins in regulating cell growth and differentiation.

### Major Findings:

#### The bZIP motif: Biochemistry

1. Novel dimerization partners of bZIP members have been determined. Using simple interhelical salt bridge rules, we have predicted novel dimerization partners for several bZIP molecules. We suggested that the bZIP molecules ATF4 and IGEBP1 would form heterodimers. We have requested and received plasmids that encode these proteins from the researchers who initially cloned these molecule and have generated pure samples of both proteins. As predicted, they prefer to form heterodimers. We are presently quantitating our results. Additional experiments suggest that ATF4 can form heterodimers with three of the C/EBP family of bZIP molecules.
2. A "Dominant Negative" form of C/EBP can be generated. Again, using simple rules of interhelical salt bridge formation, we have designed derivative molecules of C/EBP that dimerize with wild type C/EBP better than C/EBP interacts with itself. This result suggests that we are developing a rich understanding of the rules that govern dimerization specificity. Biologically, the intriguing possibility is that we will be able to introduce these "dominant negative" molecules into cells and disrupt normal C/EBP function. These studies are presently being continued.
3. Leucine zipper dimerization is enthalpically driven. We are using biophysical methods to determine the nature of the forces that are important for dimer formation. These experiments are being conducted using an analytical ultracentrifuge in collaboration with Mark Lewis at the NIH. Our initial studies, using a 63 amino acid recombinant protein show that this molecule is in a monomer-dimer equilibrium with a dimer dissociation of 10  $\mu$ M. An analysis of dimerization vs. temperature suggests that the driving force for dimer formation is enthalpic. We plan on continuing these studies with our designed molecules that have different dimerization specificities. Initial calorimetric studies with Kelly Thompson in Ernesto Friere's group at Johns Hopkins University confirm and compliment the analytical ultracentrifuge data.
4. Which interhelical salt bridge is the strongest? We are very interested in exploring which amphipathic helix can interact with endogenous amphipathic helices with the most specificity. These molecules should be the most valuable as dominant-negative reagents. To this end we are initially determining which interhelical salt bridge is the best in regulating dimerization specificity. We have generated a large systematic collection of bZIP protein mutants with different amino acids in the *e* and *g* positions. We are able to purify large quantities of these proteins enabling biophysical characterization of their properties.

#### The bZIP motif: Biology

1. We are exploring the value of our designed dominant-negative molecules in two different biological settings. The first is to use stable cell transformants. We have generated a trans-dominant protein that contains a dimerizing helix that preferentially interacts with all the CEBP family members. To this helix, we have attached the plant bZIP basic region, a bZIP basic region that binds the most

divergent DNA's of any of the known bZIP proteins. This protein heterodimerizes with CEBP family members and binds a chimeric DNA sequence. We have attached this protein to the estrogen binding domain to generate an inducible protein; in the absence of estrogen, the protein chimera is in the cytoplasm and in the presence of estrogen, the protein is translocated to the nucleus. We are presently collaborating with Ken Zaret at Brown University who is studying liver development. Dr. Zaret has a cell system that shows an inducible CEBP binding site in both the promotor and enhancer of the albumin gene. More importantly, they have an *in vivo* footprint of this transcription factor. We are using their cell system to generate stable cell transformants and with their help will use the *in vivo* footprint as an assay that our trans-dominant is displacing CEBP *in vivo*.

2. The second biological context we will study in these trans-dominant is in transgenic mice. Because of our fear that our trans-dominant protein could be dominant lethals, we have decided to use the non essential tissue, breast, as our test system. We know that three CEBP family members are expressed in breast and plan on using both the MMTV promoter and the milk protein promoter to drive expression of our various trans-dominant designs. We hope this will be a convenient system to study our various ideas concerning what makes a good trans-dominant. The obvious benefit is that we may generate valuable model systems to study breast development.

#### Publications:

Vinson C, Hai T, Boyd S. Dimerization specificity of the leucine zipper containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev* 1993; 7:1047-58.

Thompson K, Vinson C, Freire E. Thermodynamic characterization of the structural stability of the bZIP transcription factor GCN4. *Biochemistry* 1993, in press.

#### Chapter Reviews:

Baxevanis A, Vinson C. Interactions of coiled coils in transcription factors: where is the specificity? *Current Opinions in Genetics and Development* 1993;3: 278-85.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05272-02 LB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Structure and Function of RNA Polymerase II

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

M. Mortin	Senior Staff Fellow	LB	NCI
L. Burke	BTP	LB	NCI
T. Jones	Biologist	LB	NCI
M. Hutchings	Microbiologist	LB	NCI

## COOPERATING UNITS (if any)

Y. Chen and A. Greenleaf, Duke University; D. Price, University of Iowa

## LAB/BRANCH

Laboratory of Biochemistry, DCBDC

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.40

## PROFESSIONAL:

2.00

## OTHER:

.40

## 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.)

We are using a genetic approach to define the role of RNA polymerase II in regulating gene expression. Our experiments have focused on the structure of this multimeric enzyme composed of approximately 12 subunits. Four of these subunits are now cloned in *Drosophila* and three have been mutated. The vast majority of mutations that exist are in the two largest subunits (215 kd and 140 kd) that constitute greater than 70% of the enzyme's mass. Many of the mutations we have identified in the two largest subunits cause discrete mutant phenotypes suggesting that they are defective in only a subset of the functions or steps required for transcription by RNA polymerase II.

The main thrust of our research during the past year has been to begin sequencing the various mutations mentioned above and to ask if they identify specific domains of the proteins that can then be ascribed particular functions by virtue of the mutant phenotypes they elicit. In collaboration with Yan Chen and Arno Greenleaf of Duke University, we have found that mutations that interact with the transcription factor, *Ubx*, cluster in the central region of the largest subunit and the C-terminus of the second largest subunit. The mutation conferring alpha-amanitin resistance in the largest subunit and a putative elongation defective mutation also map to the central region of the largest subunit. Finally, eight mutations in the second largest subunit suppress the lethality of the putative elongation defective mutation in the largest subunit and map to the central region of this subunit. These eight mutations define two genetic groups. Five of the eight are strong suppressors and all change a serine to a cysteine. Three are weak suppressors and map 7 amino acids away, changing a methionine to an isoleucine (2) or valine. This highly conserved region identifies a domain that displays sequence similarity to the disorganized region of bacterial DNA polymerase I and suggests models for the functional and structural relationship of the two largest subunits of RNA polymerase II.



## Project Description

### Objectives:

1. To determine the structural constraints on the region identified by suppressor mutations in the second largest subunit of RNA polymerase II and to begin to define the function of this region.
2. To complete the cloning of the S3 suppressor locus and to make headway on the cloning of the lethality causing mutation associated with the S8 suppressor mutation.
3. To develop an in vitro transcription system using mutationally altered RNA polymerase II.

### Major Findings:

During the past year we have made substantial progress in analyzing two sets of mutations: those that cause the homeotic transformation related to the Ultrabithorax locus, which encodes a transcription factor, and those that suppress a mutation in the largest subunit of RNA polymerase II, which is thought to cause an elongation defective. Sequence analysis of both sets of mutations confirms that mutants within a given phenotypic class identify discrete domains within the protein.

Mutations that cause the Ubx effect cluster in the central region of the largest and the C-terminal domain of the second largest subunits. This project was a collaboration with the Greenleaf laboratory and a paper describing it has been accepted for publication in MCB. Two other Genetics papers resulted from this collaborative effort during the past year. Both describe the initial cloning and genetic identification of the second largest subunit of RNA polymerase II. One was in press at the time of our last review but has since been published, the other is currently in press.

Sequence analysis of eight suppressor mutations in the second largest subunit of RNA polymerase II that rescue a conditional lethal mutation in the largest subunit, which is thought to identify a domain required for correct elongation, identify a single domain. Furthermore, the suppressor mutations fall into two classes, strong and weak, with the strong all caused by the same serine to cysteine amino acid substitution and the weak all mapping seven amino acids away in a methionine, two changing it to an isoleucine and one to a valine. This remarkable observation has been confirmed by PCR amplification of the region surrounding the putative site of the weak suppressor, subcloning this region into a rescue construct, transformation of this construct into flies and a resulting suppression.

Comparisons of the sequence surrounding the suppressor mutations with other sequences available on computer data bases have resulted in two significant observations, currently being written up for publication. The first is that the suppressor mutations in the second largest subunit of RNA polymerase II substitute conserved amino acids that are often found in RNA polymerase I and III. The second is that the region surrounding the suppressor mutations has significant sequence identity with bacterial DNA polymerase I. The crystal structure of DNA polymerase I is available and we have used this data to place both the suppressor mutations and the conditional mutations they suppress on the tertiary structure of DNA polymerase I. Both sets of mutations, the suppressors and the conditional lethals, map in the region identified as the active domain of DNA polymerase I. Furthermore, the suppressor mutations map to a region called the disorganized region, which is thought to facilitate translocation of the DNA template during the polymerization reaction. This model is consistent with our hypothesis that the original conditional lethal mutation is defective in elongation. Progress has also been made on the cloning of the S3 locus. The 30kb walk surrounding the insertion site of a transposable element induced mutation was screened for RNAs in a reverse Northern. Radioactively labeled cDNA was used to probe the walk and demonstrated that only the 6.4kb fragment containing the transposable element encodes a detectable embryonic RNA. Northern probed with this fragment revealed a single

approximately 3kb transcript that is moderately abundant. We are currently synthesizing a putative rescue construct with a 16kb genomic clone surrounding the RNA encoding region in order to confirm our identity of this fragment as containing the S3 locus. From there it should be straightforward to isolate cDNA clones for sequence analysis.

Publications:

Mortin MA, Zuerner R, Berger S, Hamilton BJ. Mutations in the second-largest subunit of *Drosophila* RNA polymerase II interact with Ubx, *Genetics* 1992;131:895-903.

Hamilton BJ, Mortin MA, Greenleaf AL. Reverse genetics of *Drosophila* RNA polymerase II: identification and characterization of RpII140, the genomic locus for the second-largest subunit, *Genetics* 1993, in press.

Chen Y, Weeks J, Mortin MA, Greenleaf AL. Mapping mutations in genes encoding the two large subunits of *Drosophila* RNA polymerase II defines domains essential for basic transcription functions and for proper expression of developmental genes, *Mol Cell Biol* 1993, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER Z01 CB 05273-02 LB
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PERIOD COVERED  
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Molecular Genetics of Sexual Dimorphism

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

D. H. Hamer	Chief, Gene Structure and Regulation Section	LB	NCI
N. Hu	Visiting Fellow	LB	NCI
J. Zeng	Visiting Fellow	LB	NCI
A. Pattatucci	NRC Fellow	LB	NCI
B. Prickrill	IRTA	LB	NCI
S. Hu	Chemist	LB	NCI
V. Magnuson	IRTA	LB	NCI
R. Gorski	Sabbatical	LB	NCI

COOPERATING UNITS (if any)  
Jeremy Nathans, Johns Hopkins Medical School; Larry Charnas, Human Genetics Branch, NICHD; Elliot Gershow, Clinical Genetics Branch, NIMH

LAB/BRANCH  
Laboratory of Biochemistry, DCBDC

SECTION  
Gene Structure and Regulation Section

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

TOTAL MAN-YEARS: 7.75	PROFESSIONAL: 6.75	OTHER: 1.00
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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 unrounded type. Do not exceed the space provided.)

The molecular basis for the sexual differentiation of neural structure and function is being investigated in three organisms. In humans, the possible role of heredity in sexual orientation is probed by pedigree analysis, candidate gene screening, and molecular linkage studies. A correlation between male sexual orientation and the inheritance of DNA markers on chromosomal region Xq28 has been detected within a subpopulation of families in which there are two homosexual brothers. This candidate locus is distinct from the androgen receptor locus at Xq13. In *Drosophila*, attempts to clone the fruitless locus, which leads to male-male courtship behavior, have been initiated. In rat, mRNA has been prepared from a sexually dimorphic region of the hypothalamus and is being screened for sex-specific and androgen-dependent differences.

## Project Description

Objectives:

To identify genes that contribute to the differentiation of sexually dimorphic neuroanatomical structures and behavioral traits, and to understand how the gene products are regulated during development and integrated into the neural circuitry.

Major Findings:A. Human Studies

Human sexual orientation is variable. Although the majority of individuals display a heterosexual preference for members of the opposite gender, a significant minority exhibit a homosexual orientation toward members of the same sex. The purpose of our human studies is to determine whether or not this variation is influenced by heredity, and if so to isolate and characterize the relevant genes.

1. Pedigree Analysis. Traits that are genetically influenced tend to aggregate in families. We have collected family histories of sexual orientation from 114 men and 230 women who identify themselves as predominantly or exclusively homosexual. A preliminary analysis of the data leads to three conclusions: (1) Increased rates of homosexual orientation are found in the siblings of both men and women; (2) the factors responsible for this familial aggregation are at least partially distinct for males versus females; gay men have more homosexual brothers than sisters, whereas lesbians have more homosexual sisters than brothers; and (3) for men, increased rates of homosexual orientation are also observed in maternal uncles and the sons of maternal aunts, but not in paternal uncles, the other three types of cousins, or fathers. In several cases, this pattern of maternal transmission of male homosexuality is observed in three or more generations. Because uncles and cousins are raised in different households and have different mothers than the probands, these data favor an interpretation of genetic transmission rather than environmental or maternal effects. The observed excess of maternal transmission is consistent with X-linked inheritance.

2. Linkage Analysis. If a trait is genetically influenced, then related individuals who share the trait should share the gene more often than expected by chance alone. Such associations can be detected by DNA linkage studies on families in which there are two or more individuals who express the trait of interest. We have initially focused on families in which there are two gay brothers for several theoretical and practical reasons: (1) The sib-pair method is nonparametric and independent of gene penetrance and frequency; (2) a major gene can be detected even if a polygenic or environmental cofactor is required; (3) "false negatives" (individuals who have a homosexual orientation but choose to identify as heterosexual) are irrelevant; (4) "false positives" (heterosexuals who choose to identify as homosexual) are expected to be rare; and (5) it was easier to recruit sib-pairs than extended families.

To date, we have tested the inheritance of 22 X-linked polymorphic markers in 40 pairs of gay brothers. This has allowed us to exclude approximately 70% percent of the X-chromosome from playing a major role in sexual orientation in most families. However, an association between homosexual orientation and the coinheritance of polymorphic markers on chromosomal region Xq28 has been detected in approximately 65% of the families tested. With the current sample size, the probability that the linkage is genuine is given by  $P = 0.00001$ , multipoint LODmax = 4.0. These statistics give an overall confidence level of >99%, which by convention is considered to be strong evidence for linkage. However, as with all studies of this sort, confirmation of the results on an independent sample will be critical. Interestingly, the Xq28 candidate locus falls in a region of both DNA sequence repeats and homology between the X and Y chromosomes. Either unequal crossing over between duplicated sequences or exchanges between the sex chromosomes could generate sequence variants at a high rate, thereby permitting the genetic transmission of a trait that decreases the reproductive rate.

3. Karyotype and Candidate Gene Analysis: G-banded karyotypes of 24 gay men, 8 lesbians, and 7 heterosexuals have been analyzed at the 400-band level of resolution. To date, no obvious deletions, translocations or other structural variations have been observed. In addition, the presence or absence of DNA sequences encoding the testis-determining factor gene SRY, which is the primary sex-determining gene in humans, was assayed by PCR amplification in 211 gay men and 18 lesbians. No deletions or insertions were observed.

The androgen receptor plays a central role in male sexual development, and genetic males that completely lack functional androgen receptor develop as females with a typical female heterosexual orientation. In an effort to detect more subtle variations in the androgen receptor that might be associated with male homosexuality, we have collaborated with Jeremy Nathans' laboratory at Johns Hopkins Medical School to seek polymorphisms in the androgen receptor gene coding sequences. The first exon of the androgen receptor gene contains two trinucleotide repeats that vary in length in different individuals. No sexual-orientation-related differences in the average length or variability of these repeated sequences were noted in the current data base of 218 gay men. Furthermore, no DNA sequence variations that correlate with sexual orientation were found in a screen of all of the androgen receptor coding sequences in an additional 44 men, and no linkage between the androgen receptor locus and sexual orientation was found in 39 families with two homosexual brothers. Taken together, these data show that the androgen receptor locus is not a major source of variation in male sexual orientation.

4. Medical Studies: Sexually active homosexual men are at increased risk for infection by HIV, the causative agent of AIDS. An unusual feature of HIV progression in gay men is the high rate of Kaposi's sarcoma, a cancer that is found only rarely in seropositive drug users, hemophiliacs, or women. The identification of host genes that affect this response to HIV infection could lead to new therapeutic approaches to the AIDS epidemic. To date, we have accrued DNA samples from 48 HIV+ individuals, of whom nine have homosexual brothers, three have HIV+ brothers, and three have Kaposi's sarcoma. Although

the current sample is too small for an independent linkage study, it should provide an increasingly valuable resource as HIV-progression candidate loci are identified by genetic association studies that are currently being conducted on large cohorts. Our samples will be made available to the NCI in Frederick and to other research groups that are conducting these studies.

Both gay men and lesbians have been reported to be at increased risk for alcoholism and substance abuse. Through the Whitman Walker Alcohol and Substance Abuse Program and other sources, we have accrued DNA from 51 subjects with alcoholism (MAST and DSM-III-R criteria), substance abuse (primarily cocaine and opiates), or multiple addictions. Strikingly, over 90 percent of these subjects report alcoholic or addicted first-degree relatives, and DNA samples have been collected from 11 families in which alcoholism appears to be segregating. Following several reports of an association between alcoholism and an RFLP of the dopamine D2 receptor gene, we performed linkage analysis on four of these families that were informative for the polymorphism; but in agreement with others, we found no evidence for linkage. We are currently collaborating with Dr. Elliot Gershon's laboratory (NIMH) to screen our samples for point mutations or other polymorphisms in the D2 receptor gene.

#### B. Courtship Behavior in Drosophila

The fruitfly *Drosophila melanogaster* displays an elaborate and stereotypical courtship ritual that involves chemosensory, auditory and visual components. The fruitless mutation is a complex chromosomal inversion that disrupts normal courtship patterns and leads to extensive male-male courtship. We are attempting to clone the fruitless locus by a chromosomal walk from a nearby marker.

#### C. Sexually Dimorphic mRNAs in the Rat Hypothalamus

In rats, adult sexual behavior is profoundly influenced by the activity of gonadal steroids during a short, critical period of prenatal and postnatal development. Neuroanatomical studies have revealed that the sexually dimorphic nucleus of the preoptic area of the hypothalamus (SDN-POA) is an important target for neurosteroid action during this period. We are attempting to clone sexually-dimorphic and/or androgen-regulated mRNAs from this region by using an RT/PCR differential display method. Hypothalamic mRNA has been prepared from normal male, normal female, castrated male, and castrated testosterone-injected animals and screened using 82 different primer combinations. Several candidate mRNAs have been identified and are being cloned and characterized.

#### Publications:

Casas-Finet JR, Hu S, Hamer D, Karpel RL. Characterization of the copper- and silver-thiolate clusters in N-terminal fragments of the yeast ACE1 transcription factor capable of binding to its specific DNA recognition sequence, *Biochemistry* 1992;21:6617-26.

Macke JP, Hu N, Hu S, Bailey M, King VL, Brown T, Hamer D, Nathans J. Sequence variation in the androgen receptor gene is not a common determinant of male sexual orientation, *Am J Human Genet* 1993, in press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05274-02 LB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

IAP Proviruses as Multilocus Probes for Mapping on Mouse Chromosomes

## 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. Ruff                          Chief, Biosynthesis Section              LB NCI

## COOPERATING UNITS (if any)

Drs. V. Letts and W.N. Frankel, The Jackson Laboratory.

## LAB/BRANCH

Laboratory of Biochemistry, DCBDC

## SECTION

Biosynthesis Section

## INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.6

## 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 IAP LS oligonucleotide probes, derived from IAP elements that are expressed in normal mouse lymphocytes, detect restriction fragments which have characteristic strain distribution patterns (SDPs) among different strains of mice, making them suitable as multilocus probes for genome mapping. In HindIII digests, LS element probes each react with a limited number of restriction fragments that represent junctions between proviral and flanking DNA. Previously we determined chromosome assignments for 44 IAP LS proviral loci by comparing their SDPs with those of previously typed genetic markers in the C57BL/6 X DBA/2 (BXD) and BALB/c X C57BL/6 (CXB) recombinant inbred (RI) mouse strains. Since over 60% of provirus integration sites are generally shared between inbred strains of mice, mapping in these common stocks provides data for less common strains with genetically determined phenotypes of interest. We have mapped IAP proviral elements that were not polymorphic among the inbred strains in an interspecific backcross between C57BL/6J and Mus spretus in collaboration with Drs. Verity Letts and Wayne Frankel, The Jackson Laboratory. Chromosomal locations have been assigned to 70 LS proviral loci in the C57BL/6J strain. Fourteen proviral loci defined by probes based on IAP elements expressed in plasmacytomas have also been mapped in the interspecific backcross.

## Project Description

Objective:

To develop and use multilocus probes to map IAP proviruses on mouse chromosomes.

Major Findings:

Cosegregating markers in rodent genomes have been used to define genes that play a role in susceptibility to intestinal tumors, hypertension, and type I diabetes. Taking advantage of the large regions of synteny between the mouse and human genomes, studies using animal models are useful for defining comparable mutations that play a role in human disease. Subclass specific oligonucleotide probes for the IAP gene family have been developed in our laboratory for genomic mapping. We determined chromosomal assignments for 44 IAP proviral loci, defined by sequences found in IAP elements expressed in LPS-stimulated lymphocytes (lymphocyte specific or LS elements), in recombinant inbred (RI) mouse strains. For the LS1 and LS3 oligonucleotide probes, 40-60% of the loci were polymorphic between inbred strains, while for the LS2 probe 60-80% of the loci were polymorphic. An LS1 provirus, designated Iapls1-10, which cosegregated with resistance to plasmacytoma induction, has been mapped to distal chromosome 4. Two genes conferring resistance to plasmacytoma induction have also been mapped to this region by Dr. Beverly Mock, Laboratory of Genetics, NCI.

IAP proviral elements that were not polymorphic among progenitor strains used in construction of RI strains have been mapped in an interspecific backcross between C57BL/6J and an inbred line of Mus spretus, SPRET/Ei, in collaboration with Drs. Verity Letts and Wayne Frankel, The Jackson Laboratory. This panel of backcross animals has been established as a resource for the mouse genome mapping community for analysis of cDNAs. The rate of polymorphism between markers in the inbred strains and Mus spretus exceeds 80% because of the evolutionary distance between them. Few LS1 or LS3 proviruses are detectable in Mus spretus genomic DNA. There are approximately equal numbers of LS2 proviruses in the spretus and musculus genomes, but polymorphisms among the restriction fragments permit mapping of C57BL/6 proviruses. We have assigned chromosomal locations to 70 LS IAP proviral loci. Assignments have also been completed for 14 IAP loci defined by probes based on variations in sequence of IAP elements expressed in plasmacytomas.

We have also demonstrated the general utility of subclass multilocus oligonucleotide probes based on expressed genes of repetitive sequence families for genome mapping by developing subclass-specific probes from the endogenous VL30 proviral gene family.

Publications:

Lueders KK. Mapping of GLN retrotransposon LTR sequences in the BXD RI series, Mouse Genome 1990;436-8.

Lueders KK, Frankel WN, Mietz JA, Kuff EL. Genomic mapping of intracisternal A-particle proviral elements, Mammalian Genome 1993;4:69-77.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08212-19 LB
PERIOD COVERED October 1, 1992 to September 30, 1993		
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	Chief, Genes and Gene Products Section	LB NCI
R. E. Manrow	Senior Staff Fellow	LB NCI
Mi-Li Gu	Visiting Fellow	LB NCI
H.Y. Lee	Visiting Fellow	LB NCI
P. Mol	Visiting Fellow	LB NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Biochemistry, DCBDC		
SECTION Genes and Gene Products Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 4	PROFESSIONAL: 4	OTHER:
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      C		
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.) Prothymosin alpha is post-translationally modified. When human myeloma cells were metabolically labeled with [ <sup>32</sup> ]orthophosphoric acid, they synthesized [ <sup>32</sup> ]prothymosin alpha. Thin layer electrophoresis of partially hydrolyzed labeled protein indicated that serine residues were phosphorylated. Analysis of peptides derived from bovine and [ <sup>32</sup> ]human prothymosin alpha revealed that a 14-mer derived from the amino terminus was phosphorylated at a single position despite the presence of three closely spaced serine residues. Approximately 2% of the peptide in each case contained phosphate. Positive identification of the phosphorylated amino acid was obtained by colliding the 14-residue phosphopeptide with helium in the mass spectrometer and finding phosphate only on the N-terminal acetylserine residue. In a synchronized population of human myeloma cells, phosphorylation occurred throughout the cell cycle. Although prothymosin alpha is known to be essential for cell division, the constancy of both the amount of the protein and the degree of its phosphorylation suggests that prothymosin alpha does not govern mitosis. Prothymosin alpha pre-mRNAs are alternatively spliced as a consequence of adjacent AG acceptor couplets (GAGGAG) at the intron 2/exon 3 boundary of the only expressed human prothymosin alpha gene. In all human cells and tissues examined, two mRNA transcripts in the ratio of 9:1, shorter form:longer form were observed. Production of the shorter mRNA, which utilizes the second AG dinucleotide, violates two consensus rules for splice site selection. The poor performance of the first AG dinucleotide cannot be explained by its position relative to other splicing signals, judging by a study of mutant genes. The GAGGAG motif in the prothymosin alpha gene has been retained by the African monkey, <u>Colobus</u> , suggesting that the ambiguity in splice-site selection confers a selective advantage. A new, simple and highly efficient method for generating mutants called Phoenix Mutagenesis has been devised.		

## Project Description

### Objectives:

The transition from quiescence to rapid growth and division is accompanied by pronounced structural and functional changes in the cell. In normal lymphocytes isolated from the peripheral circulation, the initiation of growth and division can be brought about by treatment with mitogens. Similarly, many of the same processes are observed in growth arrested cells upon release from the constraining conditions. In both cases, the cells enter the cell cycle and progress through it by means of an orderly series of reactions including enhanced protein and RNA synthesis, replication of DNA, and synthesis, activation or destruction of stage-specific proteins at designated points in the proliferative program. It is our goal to understand the processes involved in the growth of cells and their return to quiescence. Toward this end, we have focused on prothymosin  $\alpha$ , an abundant, acidic protein found only in the nuclei of proliferating cells of all types. We plan to elucidate the function of prothymosin  $\alpha$ .

### Major Findings:

An examination of cloned cDNAs for prothymosin  $\alpha$  obtained from diverse sources revealed the existence of two not quite identical molecules. The cDNA derived from SV40-transformed human fibroblasts contained a three-base pair insertion with respect to clones from primary human lymphocytes. These systems are fundamentally different. The lymphocytes were freshly isolated from the blood of normal donors, whereas the fibroblasts were long term cultured cells. The lymphocytes were normal and diploid, whereas the fibroblasts were transformed and polyploid. In addition, it was possible that the two types of prothymosin  $\alpha$  mRNA had occurred as a result of tissue specific alternative splicing. An investigation of the only expressed human prothymosin  $\alpha$  gene suggested a mechanism. The optional triplet, GAG, was found at the intron 2/exon 3

boundary in the sequence: gtaatg<sup>☆</sup>acatggcgttttctgaggag↓ where <sup>☆</sup>a represents the inferred branch point adenine residue; the underline, the polypyrimidine tract; ↓, a possible cleavage site; ag, the presumed intron terminator; and gag, a triplet providing either a closely spaced, second splicing opportunity or an exonic glutamic acid codon.

We report now that the GAGGAG sequence at the intron 2/exon 3 boundary of the prothymosin  $\alpha$  gene appears to be unique; that both AG dinucleotides are used as splice acceptors in all human cells and tissues; and that the ratio of the shorter mRNA to the longer form is universally 9:1. Our data show that the splicing event responsible for the more prevalent mRNA violates two consensus splicing rules: (i) the selected AG dinucleotide is the second, rather than the first, following the polypyrimidine tract; and (ii) it lies in an unfavorable context, namely, a purine-rich region. In addition, the exon immediately downstream does not begin with the preferred residue, G. These aberrations apparently depend on the presence of the GAGGAG motif; COS cells transfected with mutant prothymosin  $\alpha$  genes, in which the first AG dinucleotide is preceded by a C residue (CAGGAG) or followed by a GAA triplet (GAGGAA), are spliced normally. We suspect that the ambiguity in prothymosin  $\alpha$  transcripts provides a selective advantage because the African monkey, *Colobus*, whose ancestors diverged from those of *Homo sapiens* some 30 million years ago, retains the identical sequence at the boundary of intron 2 and exon 3 despite multiple mismatches nearby. This view is consistent with our failure to identify alleles of prothymosin  $\alpha$  at this locus in the 22 human genes which were examined.

To study the origin of splice site selection *in vivo* we developed a novel and simple method for introducing point mutations into genes cloned into expression vectors. In this technique, called Phoenix Mutagenesis, a new mutated plasmid arises from fragments of the old in a single process. The method makes use of restriction endonucleases that either cleave outside of their recognition sequences or within the unspecified regions of interrupted palindromes. When the overhangs at staggered ends are maximized, the ends are statistically unique resulting in a high yield of the parent plasmid upon religation of the pieces. By judicious selection of enzymes, a small fragment can be chosen for alteration. That fragment, containing the desired mutation, can either replace or compete with the wild type fragment at the ligation step in which the expression vector is reconstituted. The result is a mutated gene in an otherwise identical vector. To facilitate the generation of mutant fragments, we have also devised a two-step polymerase chain reaction method. One strand of the products of the first PCR step acts as a primer in the second PCR step; the unnecessary complementary strand does not interfere. Thus, the mutant fragment is synthesized first as a partial fragment from the site of the mutation to one end and later, as a complete fragment from end to end. The technique can be exploited in all situations where small mutations are to be introduced into large genes. When applied to the intron 2/exon 3 boundary of prothymosin  $\alpha$ , we found that multiply fragmented plasmids could be reconstituted as well as or better than plasmids cleaved at a unique site, and that the efficiency of introducing mutations using either *Bsa* I or *Pfl* M I approached 40%.

Human prothymosin  $\alpha$  has ten targets for stable phosphorylation. There are 3 serine and 3 threonine residues located near the amino terminus, threonine residues straddling the nuclear targeting signal near the carboxyl terminus, and a serine and a threonine residue located at positions 83 and 85, respectively, of the mature protein. The bovine protein has the identical constellation of amino acids with the exception of an Ala replacing Ser 83. Phosphorylated proteins from both species were studied in parallel. To determine the type of amino acid phosphorylated, [ $^{32}$ P]prothymosin  $\alpha$  was isolated from human myeloma cells, hydrolyzed, and fractionated, resulting in the identification of labeled phosphoserine; there was no evidence for phosphorylation of threonine residues. When the radioactive human protein was digested with endopeptidases and the products separated using HPLC, the label remained associated predominantly with one peptide. However, because this fragment represented a minority species, there was insufficient material for amino acid analysis. Instead, we purified larger quantities of the protein from unlabeled prothymosin  $\alpha$  isolated from bovine thymus, and obtained the requisite peptide using the labeled human peptides as a guide. A very small optical density peak of material was recovered and analyzed in the mass spectrometer in collaboration with L. Pannell (NIDDK). The results indicate that the bovine peptide was a monophosphorylated form of a 14-mer derived from the amino terminus. Precise identification of which one of 3 possible serine residues was phosphorylated was obtained by colliding the 14-residue bovine phosphopeptide with helium in the mass spectrometer and evaluating the many products. D. Sheeley (NHLBI) participated in this work. The observation that phosphate was covalently bound only to fragments containing residue #1 showed that prothymosin  $\alpha$  is phosphorylated on the N-terminal acetylserine residue. Phosphoprothymosin  $\alpha$  accounted for only 2% of the total prothymosin  $\alpha$  in either myeloma cell or bovine thymus. The low level of modification suggested that either the amount of prothymosin  $\alpha$  or the degree of phosphorylation or both might vary as a function of the cell cycle. We measured the stability of prothymosin  $\alpha$  by pulse labeling rapidly growing cells with [ $^3$ H]glutamic acid and chasing for an extended interval. Our data indicate that prothymosin  $\alpha$  was stable with a half life (measured in molecules per cell) slightly shorter than the generation time of the cells. Accordingly, it appeared all the more likely that cell-cycle dependent phosphorylation of prothymosin  $\alpha$  regulated its activity. However, when synchronized cells were pulse labeled with

[<sup>32</sup>P]orthophosphate, prothymosin  $\alpha$  became labeled equivalently at each stage of the cell cycle. Based on these findings we postulate a role exerted throughout several phases of the cell cycle which when interrupted abrogates entry into M phase.

Publications:

Sburlati AR, De La Rosa A, Batey DW, Kurys GL, Manrow RE, Pannell LK, Martin BM, Sheeley DM, Berger SL. Phosphorylation of human and bovine prothymosin  $\alpha$  in vivo, *Biochemistry* 1992;32:4587-96.

Manrow RE, Berger SL. GAG triplets as splice acceptors of last resort: an unusual form of alternative splicing in prothymosin  $\alpha$  pre-mRNA, *J Mol Biol* 1993, in press.

## SUMMARY STATEMENT

### LABORATORY OF MOLECULAR BIOLOGY

DCBDC, NCI

OCTOBER 1, 1992 to SEPTEMBER 30, 1993

The Laboratory of Molecular Biology uses genetics, molecular biology, and cell biology to study gene activity and cell behavior. Our goal is to develop new approaches to the treatment and diagnosis of cancer, AIDS and other human diseases.

#### **Immunotoxin and Recombinant Toxin Therapy of Cancer:**

For the treatment of human cancer, I. Pastan and colleagues have developed an immunotoxin, termed LMB-1, in which monoclonal B3 is coupled to LysPE38, a genetically modified form of *Pseudomonas* exotoxin (PE). LMB-1 has been approved by the FDA and is ready to enter clinical trials. A second generation recombinant immunotoxin, LMB-7, combines the variable region of the B3 antibody with PE38. This agent is very active in mice bearing human tumor xenografts, and is well tolerated by monkeys. Efforts are underway to prepare material for clinical use. New mutant forms of PE have been created which can be selectively derivatized by polyethylene glycol to reduce immunogenicity and increase survival in the blood. Several of these mutations will be subcloned into LMB-7 to see if this recombinant immunotoxin retains activity and is less immunogenic. In addition, they have begun to identify the principle immunogenic epitopes in LMB-7. A chelate of the B3 antibody has been prepared and, when labeled with <sup>111</sup>In, will image tumors in mice. A clinical grade radioconjugate is currently being prepared. Single chain immunotoxins directed at the IL2 receptor have been made and shown to cause complete regression of tumors bearing IL2 receptors in mice. One of these, anti-Tac(Fv)-PE38, is being prepared for clinical development. A new antibody that reacts with an antigen on normal prostate and prostate carcinomas has been isolated. The antibody is an IgM and the variable regions have been cloned and grafted onto a human IgG1 constant region. The possible usefulness of this antibody for the therapy and/or diagnosis of prostate cancer is being examined. Other immunotoxins directed against the EGF receptor, the erbB2 protein, the IL6 receptor, and the IL4 receptor are also being developed. They have previously proposed that a 37 kD fragment of PE (aa 280-613) translocates to the cytosol through pores in the endoplasmic reticulum. Using a cell-free system containing microsomes, direct evidence for an interaction of PE (280-613) with microsomal protein transport pores has been obtained.

#### **Development of Immunotoxins for Cancer:**

D. FitzGerald and colleagues study interactions of *Pseudomonas* exotoxin (PE) with mammalian cells. PE binds to cells via the  $\alpha$ 2-macroglobulin receptor ( $\alpha$ 2-MR). PE but not PEglu57 bound to affinity purified  $\alpha$ 2-MR. The addition of receptor associated protein (RAP) blocked PE-mediated toxicity, and did so without competing for the same binding site. Binding to the  $\alpha$ 2-M receptor on cells is followed by endocytosis of PE. Within cells PE is cleaved between arg279 and gly280 to produce an N-terminal fragment of 28 kD and a C-terminal fragment of 37 kD. A protease with this activity was prepared from beef liver

(BLP). The cleavage of PE by the BLP was optimal at pH 5.5, was inhibited by EDTA or p-hydroxymercuribenzoate and had a Km of approx 1.0 mM. When PE was cleaved by the BLP and then added to cells, cleaved toxin killed cells with greater rapidity than native PE. Cleavage of PE by BLP resembled the activity of a furin-like protease. To confirm this, PE was incubated with recombinant human furin. Furin-mediated cleavage of PE was indistinguishable from the activity seen with the BLP. The beef liver protease and furin also cleaved diphtheria toxin. In separate experiments, a new rate-limiting step in toxin action was identified. After cleavage of PE by cells, residues at the N-terminus of the 37 kD C-terminal fragment were shown to interact with an unknown cellular component in a saturable manner.

### **Monoclonal Antibodies To Cancer Cells:**

K. Chang has developed an antibody (K1) that reacts with many ovarian cancers and mesotheliomas. The antigen (CAK1) is a 40 Kd glycosylated protein attached to the cell surface by a PI anchor. To obtain the gene encoding this antigen, he has screened a lambda expression library using antibodies, isolated clones and sequenced these clones. The protein encoded by these cDNAs is a cytosolic protein that shares an epitope with CAK1, but is probably not the membrane protein. Nevertheless, this antigen is expressed in the same cells as CAK1 and may be a member of the same family. Using an alternative approach, he has recently obtained new clones which have the characteristics of the membrane form of the CAK1 antigen. Dr. Chang has also compared the reactivity of monoclonal antibody B3 with that of K1 on human lung cancers and has shown that B3 reacts with many nonsmall cell carcinomas of the lung, whereas, K1 does not. K1 reacts with mesotheliomas, whereas, B3 does not. Therefore, these two antibodies can be used in the differential diagnosis of lung tumors.

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

I. Pastan and M. M. Gottesman have continued to analyze the mechanisms of action of the multidrug transporter and have worked on the development of new strategies to circumvent multidrug resistance in cancer and to exploit molecular knowledge of the multidrug transporter to design new cancer treatments. The multidrug transporter (P-glycoprotein) has been purified to near homogeneity and shown to be an active drug-dependent ATPase of high specific activity after reconstitution into proteoliposomes. Vesicles containing P-glycoprotein capable of transport have very active P-glycoprotein kinases, and this activity is stimulated by GTP. At least one novel plasma membrane associated P-glycoprotein-kinase has been partially purified, but its role in regulating activity of the multidrug transporter has not yet been determined. Kinetic studies demonstrate that the transporter interacts with drugs within the lipid bilayer, and indirect evidence suggests that drug may be removed from both the inner and outer leaflets of the bilayer. Molecular manipulation of P-glycoprotein by analysis of point mutations and chimeras with other members of the ATP-binding cassette (ABC) superfamily of transporters has revealed multiple regions of the molecule near or within the transmembrane domains which affect substrate specificity, and has indicated the interchangeability of ABCs between *MDR1* and *MDR2*, a related transporter of unknown specificity. Function of P-glycoprotein has been explored by insertional inactivation of the *mdr1b* gene in mouse adrenal Y-1 cells, with loss of ability of these cells to secrete steroids above basal levels. They have continued to develop the *MDR1* gene as a dominant selectable marker for gene therapy. Retroviral vectors

expressing the human *MDR1* cDNA are able to confer resistance to taxol on transduced and transplanted mouse bone marrow cells, and this strategy is under consideration for gene therapy in humans to protect bone marrow during high dose chemotherapy for cancer. Two other multidrug resistant genetic systems are under development to aid in the analysis of other mechanisms of multidrug resistance: (1) A human melanoma line cross-resistant to epipodophyllotoxins (VP-16 and VM-26) and anthracyclines which has a deletion of Ala 428 in topoisomerase II; and (2) High level *cis*-platinum resistant human hepatoma and KB adenocarcinoma cells with multiple protein alterations.

### **Mechanisms of Thyroid Hormone Action in Animal Cells:**

J. Cheng and colleagues are investigating the structure and activity of the human  $\beta 1$  thyroid hormone receptor (h-TR $\beta 1$ ). To understand the molecular basis of the thyroid hormone-dependent gene regulating activity of h-TR $\beta 1$ , the structure of the thyroid hormone binding domain (HBD) was studied. Truncated h-TR $\beta 1$  fragments were analyzed by circular dichroism (CD). The spectra are compatible with the sequence analysis which predicts that HBD contains alternating stretches of  $\alpha$ -helix and  $\beta$ -sheet. A decrease in secondary structure in fragments in which the predicted  $\beta$ -strand 1 or  $\alpha$ -helix 8 was deleted, was accompanied by loss of hormone binding activity. These results are consistent with an  $\alpha/\beta$  barrel structure for the HBD of h-TR $\beta 1$ . Based on these results, they suggest a new model for h-TR $\beta 1$ , consisting of the known DNA binding domain, linked by an  $\alpha$ -helical hinge to the HBD, with the tertiary structure of an  $\alpha/\beta$  barrel.

RXR is one of the thyroid hormone receptor accessory proteins (TRAPS). They found that *in vitro* phosphorylation of TR enhanced the binding of TR to RXR $\beta$  on several TREs by 5 to 10-fold. *In vivo*, phosphorylation increased the RXR-dependent enhancement of TR transcriptional activity by 2 to 3-fold. Thus, phosphorylation is essential for modulating the activity of h-TR $\beta 1$  by RXR $\beta$ .

To understand the molecular basis of GRTH, they have studied the thyroid hormone and DNA binding characteristics *in vitro* and the function *in vivo* of the mutant receptors. The affinity in the binding of the mutant receptors to T3 correlates well with the degree of impairment of their transactivation function in HeLa cells. All of the mutant h-TR $\beta 1$ s are able to inhibit the function of transfected wild-type h-TR $\beta 1$ , indicating the h-TR $\beta 1$  mutants inhibit the function of normal TR by a dominant negative mechanism. *In vitro* DNA studies indicate that the mutant receptors show an increased tendency to form homodimer in several TREs. Furthermore, excess amounts of transfected RXR $\beta$  could not reverse the dominant negative potency of mutant TRs *in vivo*. Therefore, competition for DNA-binding most likely mediates the dominant negative potency in patients with GRTH. These findings should help provide a more rational basis for therapeutic management of GRTH.

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

Merlino and co-workers are using transgenic mouse technology to address basic questions about the role of growth factors and oncogenes in the pathogenesis of cancer and other diseases. They have developed useful animal models for a number of important human diseases. Over the last year, Merlino and coworkers have continued their study of transgenic mice overexpressing a human transforming growth factor  $\alpha$  (TGF $\alpha$ ) gene.

These mice develop hepatic carcinoma, mammary adenocarcinoma, pancreatic metaplasia and fibrosis, and a hypertrophic gastropathy resembling Menetrier's disease. Detailed molecular and genetic analysis of these lesions has demonstrated that TGF $\alpha$  plays an important role in the development of premalignant and malignant disease. In another study, Merlino and coworkers have generated transgenic mice overexpressing transforming growth factor  $\beta$  (TGF $\beta$ ) in the pregnant mammary gland, resulting in an inhibition of gland formation and milk production. These results strongly suggest an important role for TGF $\beta$  in regulating the development and function of the breast. Currently, Merlino and coworkers are generating mice that possess more than one transgenic growth factor or oncogene (double transgenic mice) to assess their interactive potential. These transgenic animals should serve as valuable molecular models for the study of the cause and treatment of a variety of human diseases.

### Regulation of Gene Activity:

A. Johnson and I. Pastan are investigating the regulation of the epidermal growth factor receptor gene by examining the activator and repressor proteins involved in the regulation. They have shown that GCF, a transcriptional repressor, is a phosphoprotein that is primarily localized in the nucleus. GCF is encoded by a 3 kilobase RNA but the cDNA also hybridizes to RNAs of 4.5 and 1.2 kilobases. A. Johnson isolated four cDNA clones from cDNA libraries that contain DNA sequence with homology to the 5' region of the GCF cDNA. The cDNAs hybridize to RNAs of 4.5 kilobases. The homologous regions of the cDNAs include only the region that encodes the DNA binding region of GCF. The remainder of the DNA sequences are dissimilar to GCF and there is no additional homology between the cDNA clones.

The genomic sequence of GCF has been compared with the cDNA sequence and a single base insertion (T at position 787) located in the cDNA. This insertion is not found in RNA as determined by reverse transcriptase-polymerase chain reaction (RT-PCR). The frameshift that occurs when the base is removed from the cDNA sequence yields an open reading frame that would translate into a protein of 85 to 90 kilodaltons. A protein of 97 kilodalton that reacts with anti-GCF was expressed using a vaccinia virus cellular expression system.

This protein was also produced *in vitro* using a coupled transcription/translation system. Two independent cDNA clones were isolated that are homologous to GCF but lack 320 base pairs at the 5' end of GCF. These clones are being tested for their effect on gene activity.

### Regulation of the *gal* Operon of *Escherichia coli*:

S. Adhya and colleagues are studying the mechanism of negative control of gene regulation at the level of transcription using the genes of D-galactose utilization in *Escherichia coli*. They have demonstrated that at least five unlinked transcriptional units on the bacterial chromosome constitute a regulon (*gal* regulon). Two of the genes (*galR* and *galS*) encode two negative regulators, two others are responsible for the high (*mgl* operon) and low affinity (*galP*) galactose transport, and the fifth one (*gal* operon) encodes the synthesis of galactose metabolizing enzymes. Four of the transcription units contain two operators,  $O_E$  and  $O_I$ . The *mgl* operon contains only  $O_E$ . GalR and GalS, which have 85% amino acid



sequence similarities, bind to the same operator(s), but do not regulate each member of the regulon in a parallel manner. GalS also autoregulates its own gene.

Biochemical analysis of the mechanism of negative control in a purified system has shown that complete repression of the two promoters of the *gal* operon requires an interaction between the  $O_E$  and  $O_I$  bound repressor molecules which generate a DNA loop encompassing the promoter segment; and that RNA polymerase remains bound to *gal* promoter DNA under conditions of DNA looping; (iii) Binding of repressor to  $O_E$  alone causes partial repression of  $P_1$  or  $P_2$  if its location is separated from  $O_E$  by an integral number of DNA helical turns. From these results they have proposed that repressor does not repress the *gal* operon by inhibiting RNA polymerase binding but locks the promoter-bound RNA polymerase in an inactive state. Repressor bound to  $O_E$  makes a direct contact to inhibit transcription. Such inhibition is further aided by forming a DNA loop around the surface of the RNA polymerase.

They have isolated and characterized three Gal repressor mutants, called Gal super-repressor (GalR<sup>s</sup>), which are noninducible in the presence of inducer galactose. The mutational changes have been located to a region of the gene which they have previously assigned to be part of the sugar binding domain by sequence comparison of similar proteins.

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

S. Garges and S. Adhya are studying how the cyclic AMP receptor protein (CRP) activates transcription in *Escherichia coli*. They have found that CRP activation of *lac* transcription not only requires protein-protein interaction with RNA polymerase, but also transmission of some signal through the DNA to the promoter. They have also shown that for this signal to be transmitted, DNA must be intact with proper Watson-Crick base-pairing.

Using both genetic and biochemical approaches, they have also found that CRP needs some accessory factors for full activation capability. One of these factors is adenylate cyclase. They have shown that when cAMP is replaced by adenylate cyclase and ATP, CRP activates transcription of the *lac* promoter 25-fold compared to 5-fold stimulation seen in the presence of cAMP. Based on these results, they have proposed that adenylate cyclase interacts with CRP during which time cAMP is translocated to CRP.

The *pts* operon encodes proteins necessary for uptake of many sugars and for activation of adenylate cyclase. They have found that the genetic regulation of *pts* is quite unusual: There are multiple promoters each with multiple initiation sites that are regulated by the presence of cAMP and CRP, the degree of supercoiling of the DNA, and by alternate sigma factors.

#### **Structure and Mechanistic Study of *E. coli* RNA Polymerase and its Role in Clinical Applications:**

D. Jin and colleagues have performed mechanistic studies on *Escherichia coli* RNA polymerase (RNAP). They has focused on studying the two kinds of nonproductive synthesis, abortive and stuttering, during transcription initiation and on identifying the sites in RNAP that is involved in these process. They also have found that one rifampicin-

resistant (Rif<sup>r</sup>) RNAP mutant, RpoB3401, has reduced affinity for UTP and overproduced abortive initiation products at the *pyrBI* promoter. They found that wild-type RNAP also produces stuttering initiation products at the *galP2* promoter. The stuttering synthesis at *galP2* was sensitive to changes in UTP concentration and was repressed by transcription factor cAMP-CRP. A different Rif<sup>r</sup> RNAP mutant, RpoB3449, dramatically reduced stuttering synthesis at *galP2*. These results indicate that the *rif*-region is important for both the abortive and stuttering synthesis in initiation.

Several antibiotics including rifampicin inhibit wild-type RNAP in bacteria. They have determined the sensitivity of *E. coli* Rif<sup>r</sup> RNAPs to these antibiotics. Different degrees of cross-resistance were found for different antibiotics.

### **Bacterial Functions Involved in Cell Growth Control:**

S. Gottesman and colleagues have been studying the role that protein degradation plays in regulating gene expression and have continued with studies on the linkages between chromosome synthesis and partition of chromosomes during cell division. Turnover of the natural substrates of the Lon ATP-dependent protease, N and SulA, has been shown to be independent of the heat shock protein DnaJ, while the activity of another substrate of Lon, the capsular polysaccharide positive regulator RcsA, has been shown to be dependent on DnaJ activity under most circumstances. These results suggest that the heat shock proteins are not an essential component of Lon-dependent degradation of natural substrates *in vivo*. The temperature sensitivity of capsule synthesis appears to be due to misfolding of RcsA *in vivo*; similar misfolding may occur in the absence of DnaJ. Studies on the regulation of *rcsA* transcription have led to the identification of a small RNA, DsrA RNA. Increased amounts of DsrA RNA lead to increased transcription of *rcsA*, apparently because the DsrA RNA participates in opening a silenced *rcsA* promoter. The histone-like protein, HNS, is necessary for silencing. They have continued to investigate the *in vivo* function of the Clp energy-dependent protease. The specificity of this protease *in vivo* seems to be dictated by the ATPase subunit. They have found that ClpX, an alternate ATPase subunit, and ClpP mediate degradation of lambda O protein, as well as a number of other substrates. Another possible ATPase subunit has been found by sequence comparisons and its role in *in vivo* degradation is being examined. They have also been studying the regulatory events of the cell cycle using the *mbr* mutants, which have an alteration in DNA content per cell. They have been focusing on *mbrA*, which affects the coupling of DNA replication to cell elongation and have identified several genes required for *mbrA* to function. In addition, They have cloned *mbrA* and begun to analyze the physical structure of the gene. Further characterization of *mbrA* and its suppressors should elucidate the critical role it plays in cell cycle regulation.

### **DNA Replication *in vitro*:**

S. Wickner and colleagues have been studying the mechanism of protein folding carried out by chaperone proteins. They have been using an *in vitro* system that replicates DNA carrying the plasmid P1 origin of replication as a model system to study the function of three *E. coli* heat shock proteins, DnaJ, DnaK (the Hsp70 homologue), and GrpE. They found that DnaJ and DnaK, in an ATP-dependent reaction, activate the sequence specific DNA binding of the P1 initiator protein, RepA, by converting RepA dimers to monomers.

The monomer form binds avidly to *oriP1* DNA. Furthermore, RepA monomers bypass the requirement for GrpE as well as for DnaJ and DnaK in *in vitro* complementation assays with crude extracts of *dnaJ*, *dnaK*, and *grpE* mutant cells without the addition of purified heat shock proteins. Thus, the sole function of DnaJ, DnaK, and GrpE in plasmid P1 DNA replication is to convert RepA dimers to monomers. They have recently found reaction conditions that mimic the physiological situation. GrpE function is absolutely necessary for RepA activation *in vitro* with DnaJ and DnaK when the free  $Mg^{2+}$  concentration is maintained at a level of about  $1 \mu M$  by a metal ion buffer system. EDTA or physiological metabolites, including citrate, phosphate, pyrophosphate and ATP, all elicit the GrpE requirement. With these metal ion buffering systems, GrpE specifically lowers the concentration of  $Mg^{2+}$  required for the RepA activation reaction. The absence of  $Mg^{2+}$  blocks activation and high levels of  $Mg^{2+}$  in solution bypass the requirement for GrpE but not for the other two heat shock proteins. These results imply that GrpE facilitates the utilization of  $Mg^{2+}$  for an essential step in RepA activation.

### **Molecular Modeling:**

The main research interest of the Molecular Modeling Section is to use and develop theoretical means to study the forces that govern the structure and interaction of globular protein molecules, to predict the three-dimensional structure of these molecules, and to engineer protein molecules with improved properties. In the past year, the following were accomplished: (1) The laboratory's general-purpose, graphics-oriented programs, GEMM, GPLOT, and SPLOT, have been updated and improved. (2) They have proven by means of simple computer simulations of pure liquids, that the hydrophobicity does not arise from the hydrogen bonding property of water. (3) They have devised a theoretical scheme by which the change in stability of a protein molecule upon point mutation can be understood and estimated from the small molecule data on hydrophobicities. (4) A method was devised that identifies possible folding initiation sites of a protein molecule when only its sequence information is given and a method is being developed to predict the three-dimensional structure of these initiation sites. (5) Structural pattern recognition analysis was made on the a/b barrel motif of the protein structure. All known protein structures that have this motif can be automatically aligned using these recognized patterns. These patterns will be used in the future to spot the a/b barrel structural motif from the sequence of unknown proteins. (6) A pair of potential interchain disulfide bonding sites in the Fv fragment of the immunoglobulins was identified. These bonds are expected to stabilize the Fv fragment. These sites can be located for any immunoglobulin from the sequence alignment alone.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08000-23 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
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)		
PI:	A. Johnson I. Pastan	Expert Chief, Laboratory of Molecular Biology
Other:	H. Yamazaki	Visiting Fellow
		LMB, NCI NCI LMB, NCI
COOPERATING UNITS (If any)		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Molecular Biology		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Transcription factors regulating the expression of the epidermal growth factor receptor gene include the transcriptional repressor GCF. GCF is a phosphoprotein that is primarily localized in the nucleus. GCF is encoded by a 3 kilobase RNA but the GCF cDNA also hybridizes to RNA species of 4.5 and 1.2 kilobases. Screening of cDNA libraries with the DNA fragment corresponding to the DNA binding domain of GCF resulted in the isolation of four cDNAs with homologous sequence. The cDNAs have homology to the DNA binding domain and hybridize to a 4.5 kilobase RNA but for the most part the DNA sequences are dissimilar.</p> <p>The DNA sequence of the GCF cDNA was compared to genomic sequence and found to contain a 1 base insertion (T) at position 787. This insertion causes a frame-shift but is in frame with two other potential translation initiation codons (211 and 347). This would give a protein with a predicted size of 85 to 90 kilodaltons, which is in good agreement with the size of GCF immunoprecipitated from cells in culture. We have expressed a protein in cells using a vaccinia virus system that initiates translation at nucleotide 347. This protein is immunoprecipitable with anti-GCF and has a size of approximately 97 kilodaltons. This product can be synthesized <i>in vitro</i> using a coupled transcription/translation system.</p> <p>Two independently isolated cDNA clones have been selected from an FEM-X (melanoma) cDNA library and are homologous to GCF. Both clones lack the 320 bp at the 5' end of GCF that would give the DNA binding domain.</p>		

Major Findings:

GCF is a transcription factor that represses expression of the epidermal growth factor receptor gene. GCF is a phosphoprotein that is localized to the nucleus and phosphorylation of GCF is stimulated by okadaic acid, PMA and cAMP. GCF is encoded by a 3 kilobase (Kb) RNA species and the GCF cDNA hybridizes two addition RNAs of 4.5 and 1.2 Kb. Four cDNA clones have been isolated from cDNA libraries (FEM-X and OVCAR3) that also hybridize to 4.5 Kb RNA species. The DNA sequence homology region of these cDNAs include only the DNA binding (~300 bp). The remainder of the cDNAs (3.2 to 3.8 Kbp) are dissimilar in sequence. The nucleotide identity in the homology region is greater than 90% for all four clones.

The genomic structure of GCF has been partially determined. Examination of exon sequence has revealed a one base insertion (T) at position 787 of the cDNA. This insertion was confirmed using reverse transcriptase-polymerase chain reaction technology (RT-PCR). The frame-shift occurring when the base is deleted from the cDNA sequence yields an open reading frame (ORF) that would translate into a protein predicted to be 85 to 90 kilodalton. The ORF has two potential translation initiation codons, position 211 and 347. Expression of a protein initiating at position 347 has been achieved using a vaccinia virus expression system. This protein is immunoprecipitable with anti-GCF, has a size of 97 kilodaltons and can be synthesized efficiently *in vitro* by coupling *in vitro* transcription and translation.

Two new GCF cDNAs have been isolated by screening an FEM-X cDNA library. The sequence of these cDNAs are identical to the GCF cDNA with the exception of the 320 bp at the 5' end. Neither clone contained these sequences that give rise to the GCF DNA binding domain. These cDNAs will aid in determining the structure of GCF and its mechanism of action.

Publications:

Cadilla CL, Isham KR, Lee KL, Johnson AC, Kenney FT. Insulin increases transcription of the rat gene 33 through *cis*-acting elements in 5' flanking DNA, *Gene* 1992;118:223-9.

Kitada Y, Yamazaki H, Yasui W, Kyo E, Yokozaki H, Kajiyama G, Johnson AC, Pastan I, Tahara E. GC factor represses transcription of several growth factor/receptor genes and causes growth inhibition of human gastric carcinoma cell lines, *Cell Growth Differ* 1993;4:291-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08010-20 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Monoclonal Antibodies to Cancer Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)		
PI:	I. Pastan	Chief, Laboratory of Molecular Biology
Others:	K. Chang A. Rutherford	Visiting Associate Biologist
		NCI LMB, NCI LMB, NCI
COOPERATING UNITS (If any)		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Ultrastructural Cytochemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20829		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.1	1.1	1.0
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 un-reduced type. Do not exceed the space provided.)		
<p>           K. Chang has developed an antibody (K1) that reacts with many ovarian cancers and mesotheliomas. The antigen (CAK1) is a 40 Kd glycosylated protein attached to the cell surface by a PI anchor. To obtain the gene encoding this antigen, he has screened a lambda expression library using antibodies, isolated clones and sequenced these clones. The protein encoded by these cDNAs is a cytosolic protein sharing an epitope with CAK1 but is probably not the membrane protein. Nevertheless, this antigen is expressed in the same cells as CAK1 and may be a member of the same family. Using an alternative approach, he has recently obtained new clones which have the characteristics of the membrane form of the CAK1 antigen. Dr. Chang has also compared the reactivity of monoclonal antibody B3 with that of K1 on human lung cancers and has shown that B3 reacts with many nonsmall cell carcinomas of the lung, whereas, K1 does not. K1 reacts with mesotheliomas, whereas, B3 does not. Therefore, these two antibodies can be used in the differential diagnosis of lung tumors.         </p>		

Publications:

Chang K, Pastan I, Willingham MC. Frequent expression of the tumor antigen CAK1 in squamous cell carcinomas. *Int J Cancer* 1992;51:548-554.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08710-16 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) DNA Replication <i>in vitro</i>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)		
PI:	S. Wickner	Research Chemist LMB, NCI
Other:	D. Skowyra	Visiting Fellow LMB, NCI
COOPERATING UNITS (If any) K. McKenney and J. Hoskins, Center for Advanced Research in Biotechnology, Gaithersburg MD 20850		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Biochemical Genetics Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS: 2.0	PROFESSIONAL: 2.0	OTHER: 0.0
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 <span style="float: right;">B</span>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>We have been studying the mechanism of protein folding carried out by chaperone proteins. We have been using an <i>in vitro</i> system that replicates DNA carrying the plasmid P1 origin of replication as a model system to study the function of three <i>E. coli</i> heat shock proteins, DnaJ, DnaK (the Hsp70 homologue), and GrpE. We found that DnaJ and DnaK, in an ATP-dependent reaction, activate the sequence specific DNA binding of the P1 initiator protein, RepA, by converting RepA dimers to monomers. The monomer form binds avidly to <i>ori</i>P1 DNA. Furthermore, RepA monomers bypass the requirement for GrpE as well as for DnaJ and DnaK in <i>in vitro</i> complementation assays with crude extracts of <i>dnaJ</i>, <i>dnaK</i>, and <i>grpE</i> mutant cells without the addition of purified heat shock proteins. Thus, the sole function of DnaJ, DnaK, and GrpE in plasmid P1 DNA replication is to convert RepA dimers to monomers. We have recently found reaction conditions that mimic the physiological situation. GrpE function is absolutely necessary for RepA activation <i>in vitro</i> with DnaJ and DnaK when the free Mg<sup>2+</sup> concentration is maintained at a level of about 1 μM by a metal ion buffer system. EDTA or physiological metabolites, including citrate, phosphate, pyrophosphate and ATP, all elicit the GrpE requirement. With these metal ion buffering systems, GrpE specifically lowers the concentration of Mg<sup>2+</sup> required for the RepA activation reaction. The absence of Mg<sup>2+</sup> blocks activation and high levels of Mg<sup>2+</sup> in solution bypass the requirement for GrpE but not for the other two heat shock proteins. Our results imply that GrpE facilitates the utilization of Mg<sup>2+</sup> for an essential step in RepA activation.</p>		



Major Findings:

DnaK is a major heat shock protein of *E. coli* and the homologue of eucaryotic hsp70. It acts with two other heat shock proteins, DnaJ and GrpE in many cellular functions including DNA and RNA synthesis, cell division, proteolysis, protein phosphorylation, membrane transport, and the regulation of the heat shock response. We have been studying the mechanism of action of these three heat shock proteins in protein folding by elucidating their role in DNA replication of plasmids carrying the P1 origin, *oriP1*.

We found that DnaJ and DnaK activate the sequence specific DNA binding of the P1 initiator protein, RepA. RepA is a dimer in solution and forms a stable complex with DnaJ, containing a dimer each of RepA and DnaJ. DnaJ and DnaK, in an ATP-dependent reaction, stimulate the specific DNA binding activity of RepA by about 100-fold. We discovered that activation converts RepA dimers to monomers and that the monomer form binds with high affinity to *oriP1* DNA. The enzymatic activation can be mimicked by converting RepA dimers to monomers by urea denaturation followed by renaturation. We proposed that in normal growth conditions native proteins are identified as targets for DnaK through the specific recognition of a protein tag, which in our system is DnaJ. The RepA-DnaJ complex targets RepA for DnaK action.

The RepA monomerization reaction *in vitro* with purified proteins does not require GrpE. This was puzzling because we found that the replication of *oriP1* DNA by crude cellular extracts requires GrpE, as does replication *in vivo*. Moreover, RepA monomers bypass the requirement for GrpE as well as for DnaJ and DnaK in *oriP1* DNA replication *in vitro*. Thus, RepA monomerization is the only step in the *oriP1* DNA replication pathway that requires the heat shock proteins and that step requires all three.

We sought physiologically relevant reaction conditions where RepA activation requires GrpE so that we could study the mechanism of GrpE action. Because specificity in biochemical reactions often depends on the  $Mg^{2+}$  concentration, we systematically altered the  $Mg^{2+}$  concentration in the RepA activation reaction by the addition of EDTA. We discovered that GrpE stimulates the reaction in the presence of increasing concentrations of EDTA up to a 5:1 molar ratio of EDTA to  $Mg^{2+}$ . However GrpE does not bypass the  $Mg^{2+}$  requirement. In contrast, EDTA at a 3:1 ratio to  $Mg^{2+}$  inhibits RepA activation more than 95% in the absence of GrpE. We calculated that about 1  $\mu M$   $Mg^{2+}$  was free with the optimal GrpE-dependent conditions and about 10  $\mu M$   $Mg^{2+}$  was available with the optimal GrpE-independent conditions. Therefore, we succeeded in establishing GrpE dependent activation of RepA by lowering the level of free  $Mg^{2+}$  by a metal ion buffer system.

We found that GrpE restored RepA activation when the concentration of  $Mg^{2+}$  available for the reaction was reduced by biologically important  $Mg^{2+}$  chelating agents. Phosphate, citrate, and pyrophosphate at an appropriate excess of chelator to  $Mg^{2+}$  inhibited RepA activation by DnaJ and DnaK and the addition of GrpE promoted the reaction. High  $Mg^{2+}$  concentrations replaced GrpE. ATP, one of the most important physiological chelators of  $Mg^{2+}$ , could be used as a metal ion buffer system. ATP inhibited RepA activation without GrpE when its concentration was about 100-fold above the concentration of  $Mg^{2+}$ . GrpE or additional  $Mg^{2+}$  restored RepA activation. Thus, at appropriate concentrations, physiological metabolites with relatively low affinities for  $Mg^{2+}$  function as well as EDTA to sequester  $Mg^{2+}$  and elicit the GrpE requirement. Even without

metal ion buffering conditions, GrpE lowers the  $Mg^{2+}$  requirement from 10 to 5  $\mu M$ , and there is a range of  $Mg^{2+}$  concentrations in which this heat shock protein is required for the reaction. These results imply that the GrpE requirement is revealed when the concentration of free  $Mg^{2+}$  is low. The function of GrpE may be to facilitate  $Mg^{2+}$  utilization, possibly by increasing the affinity of DnaK or DnaK-DnaJ-RepA complex for  $Mg^{2+}$ .

Publications:

Baker T, Wickner S. Genetics and enzymology of DNA replication in *E. coli*. *Annu Rev Genet* 1992;26:447-77.

Wickner S, Skowrya D, Hoskins J, McKenney K. DnaJ, DnaK and GrpE heat shock proteins are required solely at the RepA monomerization step. *Proc Natl Acad Sci USA* 1992;89:10345-49.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08714-16 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
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)		
PI:	S. Gottesman	Chief, Biochemical Genetics Section LMB, NCI
Others:	W. Clark	Chemist LMB, NCI
	N. Trun	Staff Fellow LMB, NCI
	V. de Crecy Lagard	Visiting Fellow LMB, NCI
	D. Sledjeski	IRTA Fellow LMB, NCI
	Y. Jubete	Visiting Fellow LMB, NCI
COOPERATING UNITS (If any) M. Maurizi, Lab. Cell Biology, NCI; D. Gutnick, Tel Aviv University; M. Zylicz, Univ. of Gdansk; M. Yarmolinsky, Lab. Biochemistry, NCI; M. M. Gottesman, Lab. Cell Biology, NCI; M. Couturier, Universite Libre de Bruxelles; A. Toussaint, Universite Libre de Bruxelles; J. Rouviere-Yaniv, Institut de Biologie Physico-chimique		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Biochemical Genetics Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS: 5.3	PROFESSIONAL: 4.3	OTHER: 1.0
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"/> (e1) Minors <input type="checkbox"/> (e2) Interviews <span style="float: right;">B</span>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>We have been studying the role that protein degradation plays in regulating gene expression and have continued with studies on the linkages between chromosome synthesis and partition of chromosomes during cell division. Turnover of the natural substrates of the Lon ATP-dependent protease, N and SulA, has been shown to be independent of the heat shock protein DnaJ, while the activity of another substrate of Lon, the capsular polysaccharide positive regulator RcsA, has been shown to be dependent on DnaJ activity under most circumstances. These results suggest that the heat shock proteins are not an essential component of Lon-dependent degradation of natural substrates <i>in vivo</i>. The temperature sensitivity of capsule synthesis appears to be due to misfolding of RcsA <i>in vivo</i>; similar misfolding may occur in the absence of DnaJ. Studies on the regulation of <i>rscA</i> transcription have led to the identification of a small RNA, DsrA RNA. Increased amounts of DsrA RNA lead to increased transcription of <i>rscA</i>, apparently because the DsrA RNA participates in opening a silenced <i>rscA</i> promoter. The histone-like protein, HNS, is necessary for silencing. We have continued to investigate the <i>in vivo</i> function of the Clp energy-dependent protease. The specificity of this protease <i>in vivo</i> seems to be dictated by the ATPase subunit. We have found that ClpX, an alternate ATPase subunit, and ClpP mediate degradation of lambda O protein, as well as a number of other substrates. Another possible ATPase subunit has been found by sequence comparisons and its role in <i>in vivo</i> degradation is being examined. We have also been studying the regulatory events of the cell cycle using the <i>mbr</i> mutants, which have an alteration in DNA content per cell. We have been focusing on <i>mbrA</i>, which affects the coupling of DNA replication to cell elongation and have identified several genes required for <i>mbrA</i> to function. In addition, we have cloned <i>mbrA</i> and begun to analyze the physical structure of the gene. Further characterization of <i>mbrA</i> and its suppressors should elucidate the critical role it plays in cell cycle regulation.</p>		

Major Findings:

## 1. Lon:

We had predicted that the heat shock chaperone proteins such as DnaJ and DnaK might not be necessary for degradation of the naturally unstable Lon substrates such as RcsA, SulA and lambda N protein, although they are necessary for the *in vivo* recognition or degradation of abnormal proteins by Lon. This prediction has been directly tested; both SulA and N degradation by Lon is unaffected by a null mutation in DnaJ, although the Lon-independent degradation of SulA is dependent on DnaJ. RcsA is degraded less rapidly in *dnaJ* mutant cells, but this is apparently because RcsA in these cells forms insoluble, inactive aggregates which presumably are inaccessible to Lon. These observations will allow us to investigate the Lon recognition signals within the natural Lon substrates, independent of motifs which might lead to recognition of particular constructs as abnormal proteins.

## 2. Capsule synthesis:

Regulation of the capsular polysaccharide, colanic acid, in *E. coli* depends on an interaction between two positive regulators, RcsB and the unstable protein RcsA. RcsB activity is probably modified in response to appropriate stimuli via phosphorylation by the membrane sensor RcsC.

We have concentrated on the role of RcsA in capsule synthesis. In the first set of studies, derived from those described in Section 1 above, we have found that RcsA is inactive in hosts mutant for *dnaJ*, unless the other positive activator of capsule synthesis, RcsB, is in a constitutively activated form. These results are reminiscent of previous studies showing that capsule synthesis is temperature sensitive, unless a constitutively activated form of RcsB is present. In addition, while RcsA is relatively soluble at low temperature or in *dnaJ*<sup>+</sup> cells, it is insoluble in a *dnaJ* mutant. These data have led us to postulate that RcsA acts as a cellular thermometer for capsule synthesis. At elevated temperatures (or in the absence of the heat shock protein DnaJ), RcsA is unable to retain its active conformation and becomes aggregated. Increased interaction with RcsB, due to increased phosphorylation of RcsB, can protect RcsA from this aggregation and retain high levels of capsule synthesis at elevated temperatures. Purification of RcsA is proceeding to confirm some of these predictions *in vitro*.

In a separate series of experiments, we are investigating the regulation of *rcaA* transcription. *rcaA-lacZ* fusions which contain only 59 bp upstream of the transcription start are expressed at very high levels, while those with 97 bp upstream of the start are expressed at low levels. The silencing of the *rcaA* promoter by these upstream sequences is dependent on the HNS protein, a histone-like protein implicated in silencing a number of other *E. coli* promoters. For other promoters, this silencing can be overcome by specific positive regulators. In the case of *rcaA*, we have found a unique positive regulator, DsrA RNA. This small (85 nucleotides) RNA, encoded by a gene downstream of *rcaA*, can counteract the silencing effect when present on a multicopy plasmid. The effect of DsrA is not dependent on any sequences from within the *rcaA* message. Although there is no extensive region of homology between DsrA and the *rcaA* promoter, some short sequences in *rcaA* with homology to the loops formed when DsrA RNA is folded into its lowest energy form may indicate sites of interaction. Mutation in one of these regions of the *rcaA* promoter greatly

decrease the ability of DsrA to stimulate transcription; compensating mutations within *dsrA* are being tested. Similar silencing by HNS and stimulation by DsrA is seen for the *dsrB* promoter, also located downstream of the *rcaA* gene.

J. Rouviere-Yaniv (Paris) and coworkers, in collaboration with us, have found that overproduction of subunits of the histone-like protein HU also increases *rcaA* production. The overproduction of an abnormal HU may disrupt the silencing of the *rcaA* promoter or may act on the *dsrA* promoter.

### 3. Clp:

The Clp energy-dependent protease was originally identified as an activity dependent on two subunits. ClpA, an 83,000 MW protein, has two consensus nucleotide binding sites and shares extensive homology with a family of proteins found in both prokaryotes and eukaryotes. ClpP, a 23,000 MW protein carries the active site serine for the protease and is processed in a ClpP-dependent proteolytic cleavage of the N-terminal 14 amino acids.

Based on the biochemical identification by Zylicz and coworkers in Poland of an energy-dependent protease consisting of ClpP and a novel, 45,000 MW protein capable of degrading lambda O protein, we have identified the 45,000 MW protein as ClpX, encoded by the gene immediately downstream of *clpP*. ClpX has only one consensus nucleotide binding site and bears some resemblance to the C-terminal half of ClpA. Sequence analysis has suggested that yet another subfamily of ATPases with a close resemblance to ClpX exists in both gram-negative and gram-positive bacteria; we have named this new group ClpY and are investigating its possible existence in *E. coli*.

In collaboration with M. Maurizi (LCB, NCI), mutation of *clpX* or *clpP* has been shown to lead to stabilization of the lambda O protein. *clpX* mutations have no effect on the *in vivo* degradation of known ClpA-ClpP substrates, while mutations in *clpA* have no effect on degradation of O protein. These data suggest that the specificity of *in vivo* degradation is dependent on the ATPase subunit. Other groups have identified at least two other substrates specifically degraded by ClpX-ClpP.

We have constructed strains for screening for mutations which affect the ability of ClpP to function with either ClpA or ClpX, and are using these to undertake saturation mutagenesis of the *clpP* gene. Ability of the ClpAP protease to function is assayed by the level of expression of a *clpA-lac* fusion protein subject to ClpAP degradation. Loss of ClpXP protease activity is assayed by the survival of cells after loss of a plasmid containing a "plasmid addiction system" discovered by M. Yarmolinsky (LB, NCI). A protective protein or antidote encoded by this system is subject to ClpXP degradation; when the protease is intact, the antidote is rapidly degraded, allowing cell killing. When the protease is inactive, the antidote is stable and prevents cell killing. In cells deleted for the chromosomal copy of *clpP*, ClpP can be provided by a mutagenized plasmid. We have shown that the known mutations in the active sites of ClpP are defective for both ClpAP and ClpXP activities in this system; mutagenesis studies may define sites within ClpP common to both proteases, as well as any sites needed only for one of these protease activities. In addition, we have observed that ClpX overproduction, in the absence of any ClpP, leads to cell killing after plasmid loss (an indication of possible protease activity). This suggests that either ClpX alone can sequester the antidote and render it unstable, or a ClpP analog, capable of working at some level with ClpX, is present in *E. coli*. Appropriate mutant selections should distinguish between these possibilities.

#### 4. Cell Cycle Mutations:

We previously isolated mutants in *E. coli* that stably maintain twice as much DNA as wild type cells. Four loci were identified and initial characterization of the mutants, called *mbr*, suggested that *mbrB* is involved in initiation of DNA replication, *mbrC* and *mbrD* are chromosome segregation mutants and *mbrA* is a timing mutant necessary to keep DNA replication and cell elongation coupled.

We have been focusing on further characterization of *mbrA*. *mbrA* maps to 84.5 minutes on the *E. coli* chromosome and has been recently cloned onto a low copy number plasmid. Restriction mapping and subcloning of this plasmid has shown that *mbrA* is a previously unidentified gene. We are further characterizing the *mbrA* plasmid, beginning to sequence the *mbrA* gene, and using the plasmid to isolate defined null mutations.

In the course of cloning *mbrA*, we have identified at least four genes that affect *mbrA4* function. Two of these genes are required to see the phenotypes of *mbrA4* mutants: insertions in them reverse the phenotypes of *mbrA4*. Null mutations in two other known genes, *cya* and *crp*, also block *mbrA4* phenotypes. Thus, catabolite repression may either directly or indirectly regulate the expression of *mbrA*. Characterization of these genes will provide new information on the critical role of *mbrA* in the cell cycle.

#### Publications:

Gottesman S, Maurizi M. Regulation by proteolysis: energy-dependent proteases and their targets, *Microbiol Rev* 1992;56:592-621.

Conlin CA, Trun NJ, Silhavy TJ, Miller CG. *Escherichia coli prlC* encodes an endopeptidase and is homologous to *Salmonella typhimurium opdA* gene, *J Bact* 1992;174:5881-7.

Gottesman S, Clark WP, de Crecy-Lagard V, Maurizi MR. CplX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*: Sequence and *in vivo* activities, *J Biol Chem* 1993; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08750-13 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Genetic Regulatory Mechanisms in <i>Escherichia coli</i> and Its Bacteriophage		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute)		
PI:	S. Garges S. Adhya	Microbiologist Chief, Developmental Genetics Section
Others:	S. Ryu G.Rajendrakumar F. Chauvin S. Roy T. Soares	Visiting Associate Visiting Fellow Special Volunteer Special Volunteer Microbiologist
		LMB, NCI LMB, NCI LMB, NCI LMB, NCI LMB, NCI LMB, NCI 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 STAFF YEARS:	PROFESSIONAL:	OTHER:
4.75	4.25	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 un-reduced type. Do not exceed the space provided.)		
<p>We are studying how the cyclic AMP receptor protein (CRP) activates transcription in <i>Escherichia coli</i>.</p> <p>(i) We have found that CRP activation of <i>lac</i> transcription not only requires protein-protein interaction with RNA polymerase, but also transmission of some signal through the DNA to the promoter. We have shown that for this signal to be transmitted, DNA must be intact with proper Watson-Crick base-pairing.</p> <p>(ii) Using both genetic and biochemical approaches, we have also found that CRP needs some accessory factors for full activation capability. One of these factors is adenylate cyclase. We have shown that when cAMP is replaced by adenylate cyclase and ATP, CRP activates transcription of the <i>lac</i> promoter 25-fold compared to 5-fold stimulation seen in the presence of cAMP. We have proposed that adenylate cyclase interacts with CRP during which time cAMP is translocated to CRP.</p> <p>(iii) The <i>pts</i> operon encodes proteins necessary for uptake of many sugars and for activation of adenylate cyclase. We have found that the genetic regulation of <i>pts</i> is quite unusual: There are multiple promoters each with multiple initiation sites that are regulated by the presence of cAMP and CRP, the degree of supercoiling of the DNA, and by alternate sigma factors.</p>		

Major Findings:

The cyclic AMP (cAMP) receptor protein (CRP) acts in conjunction with its ligand cAMP to regulate transcription of many operons in *Escherichia coli*. We are studying the biochemical mechanism of CRP action, including the factors that modulate CRP-mediated transcriptional regulation.

## I. Transcription activation by the cyclic AMP receptor protein (CRP).

- A. The role of DNA structure in CRP activation: We found that the integrity of the DNA between where CRP is bound and the promoter is important in CRP activation of *lac*-transcription. CRP activation gradually decreased with an increased amount of interruption in the primary structure of either of the two DNA strands. CRP was totally ineffective in activating if 2 or 4 base single-stranded gaps were introduced between the CRP binding site and the promoter. This work has been submitted for publication.

To further understand the requirement of double-strandedness of the DNA in the space between the CRP and RNA polymerase binding sites, we introduced mismatched base pairs in this region of DNA. We found that CRP cannot activate transcription in such cases, indicating that intact sugar phosphate backbone is not sufficient for activation.

- B. Interaction of CRP with other factors: In a purified *in vitro* transcription system, CRP alone is sufficient for transcription activation, but the level of activation is only about 10% that of the *in vivo* activation. We have been exploring whether there are other missing factors which contribute to transcription activation by CRP. This idea is supported by the following observations.
1. Isolation of mutants defective in CRP activation: We have isolated a number of mutants that are defective in CRP activation of operons necessary for sugar utilization. The mutations do not map in *crp* or *cya* (the gene encoding adenylate cyclase), and are currently being characterized.
  2. Adenylate cyclase-CRP interaction: To test whether there is any direct role of adenylate cyclase in CRP mediated transcription activation, we replaced cAMP by adenylate cyclase and ATP in an *in vitro lac* transcription system containing CRP. Under these condition, adenylate cyclase is producing cAMP from ATP. We have found that there is less cAMP needed when adenylate cyclase is present than when it is absent. Furthermore, adenylate cyclase and ATP stimulate *lac* transcription 25-fold compared to the 5-fold stimulation by cAMP. We also have preliminary data from fluorescence experiments that there is interaction between adenylate cyclase and CRP. This interaction is destroyed in the presence of cAMP. Our current working model is that adenylate cyclase "delivers" cAMP to CRP, and we are continuing this study.

II. The *pts* operon of *Escherichia coli*.

The *ptsHI* operon of *E. coli* encodes the proteins necessary for the uptake of glucose and other sugars, HPr and Enzyme I. These proteins are part of a phosphotransferase system that translocates the phosphate group from phosphoenolpyruvate eventually to the sugar. The sugar is phosphorylated concomitantly with its uptake into the cell. As well as being



essential for the growth of *E. coli* on certain carbon sources, the *pts* operon and its gene products have several features that make it an attractive system for the study of gene regulation and protein-protein interactions.

- A. Regulation of the *pts* operon: Previously from *in vivo* gene fusion studies, we have found that the *pts* operon has two promoters  $P_0$  and  $P_1$  separated by about 100 base pairs. We have further characterized the entire promoter region by *in vivo* and *in vitro* studies. We found that the  $P_0$  and  $P_1$  promoters are very complex. Each promoter has alternate start sites that are determined by the presence of CRP or by the amount of supercoiling of the DNA template. Furthermore, we have found a third promoter for the *pts* operon. This promoter, about 70 base pairs upstream from  $P_0$  is very active *in vivo*, yet we cannot see expression from it *in vitro*. These results have been submitted for publication.

We have also analyzed the *pts* promoters using RNA polymerase with two types of sigma factors,  $\sigma^{70}$  and  $\sigma^{32}$  (heat shock sigma). Interestingly, the  $P_0$  promoter functions with both types of sigmas, making it rather unusual. Even when RNA polymerase- $\sigma^{32}$  is used, CRP still activates transcription. This is the first case for a transcription activator functioning with two different types of RNA polymerase.

- B. The proteins of the *pts* operon: HPr and Enzyme I are encoded by the *pts* operon. Enzyme I is active as a dimer and inactive as a monomer. In its active state, it can accept a phosphate group from PEP and transfer it to HPr. Since the physiological concentration of Enzyme I hovers around the  $K_D$  for dimerization, small changes in the amount of Enzyme I can have large effects in the cell. This makes the regulation of *pts* activity especially important (see above) and also makes the mechanisms for dimerization, that is, the contacts between the monomers interesting. In a collaboration with Dr. Saul Roseman at Johns Hopkins University, we are using site-directed mutagenesis to make Enzyme I protein that has only one tryptophan. This will allow fluorescence experiments to measure dimerization of Enzyme I under different conditions to be performed and interpreted more easily.

Previous data from Dr. Roseman's laboratory have suggested that Enzyme I has two domains. The N terminal domain is the site of phosphorylation and of interaction with HPr. We have separately cloned and expressed the N terminal and C terminal domains of Enzyme I. This will allow structural studies of the two domains independent of each other. Furthermore, we have found that the N terminal fragment acts to inhibit the phosphotransferase system *in vivo*. The mechanism for the inhibition will be explored.

### III. Guanylate cyclase in *E. coli*.

*E. coli* has guanylate cyclase activity, but nothing is known of its role. Since cGMP levels change throughout the *E. coli* cell cycle, cGMP may have a role in cell cycle. The gene encoding the *E. coli* guanylate cyclase is unknown. Identification of the gene encoding guanylate cyclase would enable the study not only of the protein, but factors controlling its synthesis and activity.

We have developed a genetic screen for guanylate cyclase mutants. Previously, we isolated mutants of the *crp* gene encoding the cAMP receptor protein. These mutants, called CRP\*, could function *in vivo* in the absence of cAMP, but *in vitro* required cAMP or, unlike wild type CRP, cGMP. We reasoned that some of these CRP\* mutants *in vivo* were using cGMP as the ligand to activate the CRP. Therefore, starting with these mutants, we have isolated mutations that prevent these CRP\* mutants from activating transcription. These mutants are correctable by the addition of exogenous cAMP or cGMP. These mutations have been mapped to three different loci, and are currently being characterized. We postulate that these mutants are affected in the synthesis or functioning of cGMP.

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Kim J, Adhya S, Garges S. Allosteric changes in CRP: Hinge reorientation, Proc Natl Acad Sci USA 1992;89:9700-4.

Ryu S, Kim J, Adhya S, Garges S. Pivotal role of amino acid at position 138 in the allosteric hinge reorientation of cAMP receptor protein, Proc Natl Acad Sci USA 1993;90:75-9.

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Kolb A, Busby S, Buc H, Garges S, Adhya S. Transcriptional regulation by cAMP and its receptor protein, Ann Rev Biochem 1993; in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08751-13 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of the <i>gal</i> Operon of <i>Escherichia coli</i>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute)		
PI:	S. Adhya	Chief, Developmental Genetics Sect. LMB, NCI
Other:	H. Choy	Visiting Associate LMB, NCI
	S. W. Park	Visiting Fellow LMB, NCI
	Y. N. Zhou	IRTA Fellow LMB, NCI
	M. Geanacopoulos	IRTA Fellow LMB, NCI
	P. Parrack	Visiting Fellow LMB, NCI
	S. Garges	Microbiologist 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 STAFF YEARS: 5.75	PROFESSIONAL: 5.75	OTHER:
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		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>We are studying the mechanism of negative control of gene regulation at the level of transcription using the genes of D-galactose utilization in <i>Escherichia coli</i>. We have demonstrated that at least five unlinked transcriptional units on the bacterial chromosome constitute a regulon (<i>gal</i> regulon). Two of the genes (<i>galR</i> and <i>galS</i>) encode two negative regulators, two others are responsible for the high (<i>mgl</i> operon) and low affinity (<i>galP</i>) galactose transport, and the fifth one (<i>gal</i> operon) encodes the synthesis of galactose metabolizing enzymes. Four of the transcription units contain two operators, <i>QE</i> and <i>QI</i>. The <i>mgl</i> operon contains only <i>QE</i>. GalR and GalS, which have 85% amino acid sequence similarities, bind to the same operator(s), but do not regulate each member of the regulon in a parallel manner. GalS also autoregulates its own gene.</p> <p>We have isolated and characterized three Gal repressor mutants, called Gal super-repressor (GalRs), which are noninducible in the presence of inducer galactose. The mutational changes have been located to a region of the gene which we have previously assigned to be part of the sugar binding domain by sequence comparison of similar proteins.</p> <p>Biochemical analysis of the mechanism of negative control in a purified system has shown that: (i) Complete repression of the two promoters of the <i>gal</i> operon requires an interaction between the <i>QE</i> and <i>QI</i> bound repressor molecules which generate a DNA loop encompassing the promoter segment; (ii) RNA polymerase remains bound to <i>gal</i> promoter DNA under conditions of DNA looping; (iii) Binding of repressor to <i>QE</i> alone causes partial repression of <i>P1</i> or <i>P2</i> if its location is separated from <i>QE</i> by an integral number of DNA helical turns. From these results we have proposed that repressor does not repress the <i>gal</i> operon by inhibiting RNA polymerase binding but locks the promoter-bound RNA polymerase in an inactive state. Repressor bound to <i>QE</i> makes a direct contact to inhibit transcription. Such inhibition is further aided by forming a DNA loop around the surface of the RNA polymerase.</p>		

Other Personnel:

G. Bouffard                      Special Volunteer                      LMB, NCI

Major Findings:Negative control of *gal* regulon.

The genes of D-galactose transport and metabolism are negatively regulated not only by Gal repressor (GalR) but also by an isomer of the repressor, termed Gal isorepressor (GalS). The galactose regulatory system constitutes a regulon. Although the two regulators act by binding to the same operator elements, the repressor and isorepressor do not regulate each member of the regulon in a parallel fashion. The following results and conclusions highlight our research during the year of 1992-1993.

1. *Purification of the isorepressor.* A T7 RNA polymerase/promoter system was used to overexpress the GalS in cells in which the *galR* gene was deleted to prevent possible co-purification. Although a large part of the overexpressed protein formed inclusion bodies, starting with the soluble fraction of the cell, we have standardized conditions and purified the GalS protein by ion exchange chromatography, mono Q fast pressure liquid chromatography and gel filtration, in that order. Purified GalS is currently being characterized biochemically.
2. *Discovery of a new regulatory element.* During the implementation of our selection scheme to isolate bacterial mutations in the Gal isorepressor gene, we also found a mutation that increases the promoter activity of the *gal* operon by 10-fold without affecting the repression system. The mutation has been mapped at 84 min position on the *E. coli* chromosome, a region of previously unknown function. Analysis of the gene and its product are in progress.
3. *An adaptor in Gal repressor action.* The repression of the *gal* operon promoters ( $P_1$  and  $P_2$ ) requires *in vivo* an association between two repressor molecules bound to the two operators,  $O_E$  and  $O_I$  forming a DNA-loop. Using a unitary promoter plasmid as a template, we have shown that repression by Lac repressor when used in *gal* DNA in which the *gal* operators were replaced by *lac* operators is mediated by DNA loop formation *in vitro*. However, Gal repressors which bound to the two *gal* operators did not associate to form a loop and thus, as expected, fail to repress the promoters normally. We have predicted the existence of an adaptor molecule which helps the two operator-bound Gal repressors to associate and brings about repression. In order to identify the adaptor, we have set up genetic selection techniques to isolate *E. coli* mutants defective in the proposed adaptor and have isolated seven mutants with expected phenotypes. These mutants are currently being analyzed.
4. *Biochemical mechanism of repressor action.* The results of *in vitro* experiments in studying Gal repressor action have indicated that repressor-mediated DNA looping cages the RNA polymerase molecule and establishes a direct contact between repressor occupying  $O_E$  and promoter bound RNA polymerase. Such a contact inhibits the action of RNA polymerase from initiating the formation of first phosphodiester bond. Consistently, repressor when bound only to  $O_E$  brought about up to 75% repression without caging. This repression is dependent upon having the repressor bound to  $O_E$  on the same face of DNA as the RNA polymerase at the promoter.

5. *Characterization of Gal super-repressor.* We have isolated and characterized three Gal repressor mutants, called Gal super-repressor (GalR<sup>S</sup>), which are noninducible in the presence of inducer galactose. The mutational changes have been located to a region of the gene which we have previously assigned to be part of the sugar binding domain by sequence comparison of similar proteins. Two of the deduced amino acid changes, Y244F (*galR*<sub>17</sub><sup>S</sup>) and S184F (*galR*<sub>78</sub><sup>S</sup>) are among the conserved residues. The mutant genes have been cloned into overproducing vectors and one of the mutant repressors purified for studying *in vitro*. The results show that like wild type GalR<sup>+</sup>, GalR<sub>17</sub><sup>S</sup> represses transcription from the *P*<sub>1</sub> promoter more efficiently than from the *P*<sub>2</sub> promoter of the *gal* operon in the absence of CRP, and switches transcription from the *galP*<sub>1</sub> promoter to the *galP*<sub>2</sub> promoter in the presence of cAMP, but unlike GalR<sup>+</sup>, GalR<sub>17</sub><sup>S</sup> is far less sensitive to the presence of galactose for repression. As has been observed *in vivo*, GalR<sub>17</sub><sup>S</sup> is stable at both high and low temperatures, whereas, GalR<sub>78</sub><sup>S</sup> is more stable at high temperature than at low temperature.

6. *Simultaneous occupation of repressor CRP and RNA polymerase at gal promoters.* Previously, we have shown by DNase protection studies that Gal repressor, CRP and RNA polymerase each bind to the *gal* promoters without interfering with the binding of the other. We have further investigated this finding using Lac repressor and *gal* promoter fragments in which the *gal* operators were replaced by *lac* operators. These were investigated by restriction nuclease protection studies and by gel electrophoretic mobility shift experiments. Results obtained so far indicate that Lac repressor, CRP and RNA polymerase can occupy the *gal* promoter segment at the same time. These findings support our model that repressor does not inhibit *gal* transcription by sterically hindering RNA polymerase or CRP binding but by acting at a post-binding level.

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Weickert MJ, Adhya A. A family of bacterial regulators homologous to Gal and Lac repressors, *J Biol Chem* 1992;267:15869-74.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08752-13 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
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)		
PI:	Sheue-yann Cheng	Chief, Gene Regulation Section LMB, NCI
Others:	C. Parkison	Technician LMB, NCI
	M. Bhat	Visiting Fellow LMB, NCI
	J. Park	Visiting Fellow LMB, NCI
	X. Zhu	Visiting Fellow LMB, NCI
COOPERATING UNITS (if any) Peter McPhie, Laboratory of Biochemical Pharmacology, NIDDK Bruce Weintraub, Molecular, Cellular and Nutritional Endocrinology Branch, NIDDK		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Gene Regulation Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0
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		B
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)		
<p><b>I. Structure and activity of the human <math>\beta 1</math> thyroid hormone receptor (h-TR<math>\beta 1</math>).</b></p> <p><b>A. The structure of thyroid hormone binding domain (HBD):</b> To understand the molecular basis of the thyroid hormone-dependent gene regulating activity of h-TR<math>\beta 1</math>, the structure of HBD was studied. Truncated h-TR<math>\beta 1</math> fragments were analyzed by circular dichroism (CD). The spectra are compatible with the sequence analysis which predicts that HBD contains alternating stretches of <math>\alpha</math>-helix and <math>\beta</math>-sheet. A decrease in secondary structure in fragments in which the predicted <math>\beta</math>-strand 1 or <math>\alpha</math>-helix 8 was deleted, was accompanied by loss of hormone binding activity. These results are consistent with an <math>\alpha/\beta</math> barrel structure for the HBD of h-TR<math>\beta 1</math>. Based on these results, we suggest a new model for h-TR<math>\beta 1</math>, consisting of the known DNA binding domain, linked by an <math>\alpha</math>-helical hinge to the HBD, with the tertiary structure of an <math>\alpha/\beta</math> barrel.</p> <p><b>B. The essential role of phosphorylation in retinoid X receptor (RXR)-dependent activation of h-TR<math>\beta 1</math>:</b> RXR is one of the thyroid hormone receptor accessory proteins (TRAPS). We found that <i>in vitro</i> phosphorylation of TR enhanced the binding of TR to RXR<math>\beta</math> on several TREs by 5 to 10-fold. <i>In vivo</i>, phosphorylation increased the RXR-dependent enhancement of TR transcriptional activity by 2 to 3-fold. Thus, phosphorylation is essential for modulating the activity of h-TR<math>\beta 1</math> by RXR<math>\beta</math>.</p> <p><b>II. The molecular basis of generalized resistance to thyroid hormone (GRTH):</b> To understand the molecular basis of GRTH, we have studied the thyroid hormone and DNA binding characteristics <i>in vitro</i> and the function <i>in vivo</i> of the mutant receptors. The affinity in the binding of the mutant receptors to T3 correlates well with the degree of impairment of their transactivation function in HeLa cells. All of the mutant h-TR<math>\beta 1</math>s are able to inhibit the function of transfected wild-type h-TR<math>\beta 1</math>, indicating the h-TR<math>\beta 1</math> mutants inhibit the function of normal TR by a dominate negative mechanism. <i>In vitro</i> DNA studies indicate that the mutant receptors show an increased tendency to form homodimer in several TREs. Furthermore, excess amounts of transfected RXR<math>\beta</math> could not reverse the dominant negative potency of mutant TRs <i>in vivo</i>. Therefore, competition for DNA-binding most likely mediates the dominant negative potency in patients with GRTH. These findings should help provide a more rational basis for therapeutic management of GRTH.</p>		

Major Findings:I. Structure and activity of the human  $\beta 1$  thyroid hormone receptor (h-TR $\beta 1$ ).

## A. The structure of the hormone binding domain (HBD).

By analogy with steroid hormone receptors, four structural domains can be assigned to h-TR $\beta 1$ : A/B (M<sup>1</sup>-L<sup>101</sup>), C (C<sup>102</sup>-A<sup>170</sup>), D (T<sup>171</sup>-K<sup>237</sup>) and E (R<sup>238</sup>-D<sup>456</sup>). The amino terminal A/B domain is involved in activation and repression of target genes. Domain C, the only region of the molecule whose structure is understood, contains two zinc fingers which bind DNA. These are linked by domain D to domain E, which alone was thought to bind 3,3',5-triiodo-L-thyronine (T<sub>3</sub>). To understand the molecular basis of T<sub>3</sub>-dependent gene regulating activity of h-TR $\beta 1$ , we studied the structure of HBD.

Using a series of deletion mutants, we recently showed that both domain D and the eight carboxyl-terminal residues of domain E are essential for hormone binding activity. In addition, recent studies also demonstrated that the carboxyl terminal sequence of h-TR $\beta 1$  is critical for its association with one of the thyroid hormone receptor accessory proteins (TRAPs), the retinoid X receptor (RXR).

To understand the molecular basis of the critical role of these regions in the function of HBD, truncated h-TR $\beta 1$  fragments, MD32 (M<sup>169</sup>-D<sup>456</sup>), KD29 (K<sup>201</sup>-D<sup>456</sup>), DD28 (D<sup>211</sup>-D<sup>456</sup>), KD25 (K<sup>235</sup>-D<sup>456</sup>) and KP28 (K<sup>201</sup>-P<sup>448</sup>) were analyzed by circular dichroism (CD). MD32 and KD29 show intense CD spectra with double minima at 222 nm and 208-210 nm, indicating the presence of extensive regions of  $\alpha$ -helix. DD28 and KD25 have spectra which are reduced in intensity, with minima around 215 nm, characteristic of  $\beta$ -sheet. The observed spectra are compatible with sequence analysis which predicts that HBD contains alternating stretches of  $\alpha$ -helix and  $\beta$ -strand. These extensive decreases in secondary structure in DD28 and KP28 in which the predicted first  $\beta$ -strand and the last  $\alpha$ -helix was deleted, respectively, were accompanied by loss of hormone binding activity. Based on these results, we suggest a new model for h-TR $\beta 1$ , consisting of the known DNA binding domain, linked by an  $\alpha$ -helical hinge to the HBD, with a tertiary structure of an  $\alpha/\beta$  barrel. The model is compatible with previous chemical and genetic studies on the structure of this protein.

B. The essential role of phosphorylation in the retinoid X receptor (RXR)-dependent activation of the transcriptional activity of h-TR $\beta 1$ .

Recent studies have indicated that thyroid hormone receptors require TRAPs for efficient DNA binding. One of the TRAPs in HeLa cells has been identified as RXR. Transfection experiments indicate that RXR enhances the transcriptional activity of TR and T<sub>3</sub>-dependent responsiveness.

We have previously demonstrated that phosphorylation is essential for TR to bind to the thyroid hormone response elements (TREs). Furthermore, phosphorylation confers the ability of TR to interact with the TRAPs. Since RXR has been shown to be one of the TRAPs, we evaluated the role of phosphorylation of TR in the interaction of TR with RXR $\beta$ .

The effect of phosphorylation of h-TR $\beta$ 1 on its heterodimerization with RXR $\beta$  was examined by electrophoretic gel mobility shift (EMSA). Three TREs representing different orientation and spacing of the half-site binding motif, AAGGTCA, were evaluated. TREpal has the palindrome orientation of AAGGTCA with no spacing between the two half-sites. TRElap has the two half-sites in an inverted palindromic orientation. DR4 is a direct repeat of the half-sites separated by four gaps. F2 is the chicken lysozyme gene TRE. Heterodimerization of h-TR $\beta$ 1 with RXR $\beta$  was compared before and after phosphorylation. Before phosphorylation, heterodimerization of h-TR $\beta$ 1 to RXR $\beta$  when bound to F2 or TRElap was weak. After phosphorylation, heterodimerization was increased by 10-fold and 6-fold, respectively. A similar but smaller (3 to 4-fold) increase was found for DR4 and TREpal. The phosphorylation-dependent heterodimerization of h-TR $\beta$ 1 with RXR $\beta$  is reversible. When the Pi was removed by treating the phosphorylated h-TR $\beta$ 1 with either acid phosphatase or alkaline phosphatase, h-TR $\beta$ 1 could no longer heterodimerize with RXR $\beta$ . These data indicate that phosphorylation is essential for binding of h-TR $\beta$ 1 to RXR $\beta$ . These results suggest that a structural modification of TR by phosphorylation is required for heterodimerization with RXR $\beta$ . Thus, phosphorylation plays an important role for h-TR $\beta$ 1 to cross-talk with other transcriptional factors in other signal pathways.

## II. The molecular basis of generalized resistance to thyroid hormone.

The syndrome of generalized resistance to thyroid hormone (GRTH) is an inherited disease in man characterized by a resistance of the peripheral and pituitary tissues to the action of thyroid hormone (TH). The patients have elevated circulating levels of free TH and inappropriately normal or elevated levels of thyroid stimulating hormone. Refetoff *et al.* were the first to describe clinically this inherited disease in a family with deaf-mutism, stippled epiphyses, goiter, high circulating thyroid hormone levels and detectable thyroid stimulating hormone. However, the severity and symptoms of refractoriness vary considerably. There is a broad spectrum of resistance among different tissues within a given patient and different kindreds have variable patterns of tissue resistance. The mode of inheritance is autosomal dominant in the majority of the family. But recessive inheritance has also been found.

Recent isolation of the genes for TR has made it possible to test the possibility as to whether GRTH is linked to the abnormality of TRs. The two human genes encoding TRs, h-TR $\alpha$  and h-TR $\beta$ , are located on chromosome 17 and 3, respectively. h-TR $\alpha$  and h-TR $\beta$  are structurally similar in that they each contain a DNA and thyroid hormone binding domain. Each of the two genes also yields isoforms by alternative splicing. Genetics analysis of resistance kindreds has linked GRTH to the TR $\beta$  locus on chromosome 3. Mutations in one of the two alleles of the h-TR $\beta$  gene have been identified in 31 families with GRTH. This is consistent with a dominant pattern of inheritance.

Three possible mechanisms for the dominant negative action of the h-TR $\beta$ 1 mutants are: (i) formation of inactive dimers between mutant and normal h-TR $\beta$ 1, (ii) competition of normal and mutant h-TR $\beta$ 1 for binding to TRE of target genes, and (iii) competition for limiting amounts of nuclear auxiliary factors. While the first hypothesis could not be tested experimentally, we have evaluated the hypotheses (ii) and (iii).



We studied the thyroid hormone and DNA binding characteristics *in vitro* and the functions *in vivo* of the mutant receptors. Three mutant receptors, kindreds ED, PV and OK were studied. The T<sub>3</sub> binding affinity of ED and OK are 0.7 and 0.6 x 10<sup>10</sup> M<sup>-1</sup>, respectively, where of the wild type, h-TRβ1, is 3.2 x 10<sup>10</sup> M<sup>-1</sup>. Kindred PV does not bind to T<sub>3</sub>. h-TRβ1s are able to inhibit the function of transfected wild-type h-TRβ1. The affinity in the binding of the mutant receptors to T<sub>3</sub> correlates with the degree of impairment of their transactivation function. These results indicate that h-TRβ1 mutants inhibit the function of normal TR by a dominate negative mechanism.

*In vitro* DNA binding indicates that the mutant receptors showed an increased tendency to form homodimer on TRE<sub>pal</sub>, TRE<sub>lap</sub> and DR4 by 2 to 5-fold as compared to the wild type h-TRβ1. The extent of increase in homodimer formation not only depends on the kind of TRE, but also depends on T<sub>3</sub> binding affinity of the mutants. It is inversely proportional to the T<sub>3</sub> binding affinity. Furthermore, on an inverted palindrome (TRE<sub>lap</sub>), the wild type TR binding is dissociated from DNA by T<sub>3</sub>. The extent of dissociation of DNA from mutant receptors is proportional to the T<sub>3</sub> binding affinity. These results suggest that the mutant and wild type receptors compete for DNA binding.

The *in vitro* DNA binding indicates that RXRβ induced the formation of h-TRβ1:RXRβ heterodimer equally well for the mutants and the wild type h-TRβ1 on TRE<sub>lap</sub>, TRE<sub>pal</sub> and DR4. Furthermore, *in vivo* transfection in both CV1 and HeLa cells indicate that transfecting of excess RXRβ could not reverse the dominant negative action. The data demonstrate that binding of limiting amounts of endogenous nuclear RXRβ to mutant h-TRβs cannot explain the repressive action on wild type h-TRβ1 function.

These results demonstrate that the binding of mutant h-TRs to DNA as well as their dominant negative potency are TRE dependent. Assuming that different types of TREs are relevant in the various T<sub>3</sub>-regulated genes, it can be hypothesized that the dominant negative action of mutant TRs varies among different target genes and organs. In addition, competition for DNA-binding, rather than for limiting amounts of RXRβ, is likely to mediate the dominant negative action.

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Ashizawa K, Cheng S-y. A sensitive and rapid *in situ* immunoassay to quantitatively determine the cellular antigens in intact cultured cells, *J Biochem Biophys Methods* 1992;24:297-307.

Lin K-H, Ashizawa K, Cheng S-y. Phosphorylation stimulates the transcriptional activity of the human β1 thyroid hormone nuclear receptor, *Proc Natl Acad Sci USA* 1992;89:9277-81.

Ashizawa K, Cheng S-y. Regulation of thyroid hormone receptor-mediated transcription by a cytosol protein, *Proc Natl Acad Sci USA* 1992;89:7737-41.

Obata T, Cheng S-y. Regulation by thyroid hormone of the synthesis of a cytosolic thyroid hormone binding protein during liver regeneration, *Biochem Biophys Res Comm* 1992;189:257-63.

Park J-B, Ashizawa K, Parkison C, Cheng S-y. The DNA and hormone binding activity of human  $\beta 1$  thyroid hormone receptor purified from *E. coli* by immunoaffinity chromatography, *J Biochem Biophys Methods* 1993; in press.

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McPhie P, Parkison C, Lee, BK, Cheng S-y. Structure of the hormone binding domain of human  $\beta 1$  thyroid hormone nuclear receptor: Is it an  $\alpha/\beta$  barrel?, *Biochem* 1993; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08753-11 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Immunotoxins and Recombinant Toxin Therapy of Cancer		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute)		
PI:	I. Pastan	Chief, Laboratory of Molecular Biology NCI
Co-investigator:	D. FitzGerald	Microbiologist LMB, NCI
COOPERATING UNITS (if any) Molecular Oncology, Gaithersburg, MD; Kabi Pharmacia, Sweden; Cardiology Branch, NHLBI, NIH; Div. of Cytokine Biology, CBER, FDA; Div. of Virology, CBER, FDA; Protein Design Labs, Mountain View, CA; (cont. on next page)		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Molecular Biology		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
13.8	10.8	3.0
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 <input type="checkbox"/> (a2) Interviews		
		B
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>For the treatment of human cancer, we have developed an immunotoxin, termed LMB-1, in which monoclonal B3 is coupled to LysPE38. LMB-1 has been approved by the FDA and is ready to enter clinical trials. A second generation recombinant immunotoxin, LMB-7, combines the variable region of the B3 antibody with PE38. This agent is very active in mice bearing human tumor xenografts, and is well tolerated by monkeys. Efforts are underway to prepare material for clinical use. Mutant forms of PE have been created which can be selectively derivatized by polyethylene glycol to reduce immunogenicity and increase survival in the blood. Several of these mutations will be subcloned into LMB-7 to see if this recombinant immunotoxin retains activity and is less immunogenic. In addition, we have begun to identify the principle immunogenic epitopes in LMB-7. A chelate of the B3 antibody has been prepared and when labeled with <sup>111</sup>In will image tumors in mice. A clinical grade radioconjugate is currently being prepared. Single chain immunotoxins directed at the IL2 receptor have been made and shown to cause complete regression of tumors bearing IL2 receptors in mice. One of these, anti-Tac(Fv)-PE38, is being prepared for clinical development. A new antibody that reacts with an antigen on normal prostate and prostate carcinomas has been developed. The antibody is an IgM and the variable regions have been cloned and grafted onto a human IgG1 constant region. The possible usefulness of this antibody for therapy or diagnosis of prostate cancer is being examined. Other immunotoxins directed against the EGF receptor, the erbB2 protein, the IL6 receptor, and the IL4 receptor are also being developed. We have previously proposed that a 37 kD fragment of PE (aa 280-613) translocates to the cytosol through pores in the endoplasmic reticulum. Using a cell-free system containing microsomes, direct evidence for an interaction between PE (280-613) with microsomal protein transport pores has been obtained.</p>		

Cooperating Units:

Metabolism Branch, DCBDC, NCI, NIH; Upjohn, Kalamazoo, MI; Immunomedics, Newark, NJ; Hoffmann-La Roche, Nutley, NJ; Dept. of Urology, Stanford University, Stanford, CA; Nuclear Medicine, Clinical Center, NIH; Lab. Developmental and Molecular Immunity, NICHD, NIH; Biotechnology Unit, NIDDK, NIH; Dept. of Biochemistry, Pennsylvania State University, University Park, PA; Div. of Epidemiology, Statistics & Preventive Medicine, NICHD, NIH; Merck, West Point, PA; Div. of Hematology-Oncology, University of Arkansas for Medical Sciences, Little Rock, AR; Division of Cancer Treatment, NCI

Other Personnel:

I. Benhar	Visiting Associate	LMB, NCI
U. Brinkmann	Visiting Associate	LMB, NCI
J. Buchner	Visiting Fellow	LMB, NCI
A. Kihara	Visiting Fellow	LMB, NCI
R. Kreitman	Senior Clinical Associate	LMB, NCI
C.-T. Kuan	Visiting Fellow	LMB, NCI
L. Pai	Senior Staff Fellow	LMB, NCI
Y. Reiter	Special Volunteer	LMB, NCI
C. Theuer	Research Associate	LMB, NCI
Q.-C. Wang	Visiting Scientist	LMB, NCI
K. Webber	Senior Staff Fellow	LMB, NCI
M. Gallo	Microbiologist	LMB, NCI
E. Lovelace	Biologist	LMB, NCI
A. Harris	Biological Laboratory Technician	LMB, NCI

Major Findings:

The Molecular Biology Section has as its goal to develop new types of therapies for the treatment of cancer. One major project involves the use of *Pseudomonas* exotoxin (PE) attached to an antibody or growth factor as an anticancer agent. The other major project with M.M. Gottesman, LCB concerns the molecular basis of multidrug resistance in cancer.

Immunotoxins are prepared by attaching mutant forms of *Pseudomonas* exotoxin (PE) to antibody molecules that target cancer cells. Two types of immunotoxins have been made. The conventional immunotoxin consists of an antibody attached by a chemical linkage to a recombinant form of PE. The principal form of PE we have used is a 38,000 molecular weight form termed PE38. This molecule contains a deletion of domain Ia (amino acids 1-252), a deletion of a portion of domain Ib (amino acids 365-380) and a lysine containing peptide extension at the amino terminus. This lysine is used to couple the LysPE38 to antibodies. The principle monoclonal antibody under development is termed MAb B3 and it reacts with many human cancers including cancers of the colon, breast, ovary, lung, stomach, esophagus, bladder and prostate. This antibody also reacts with some normal tissue especially the glands of the stomach and the superficial epithelium of the trachea, bladder and esophagus.

To prepare material for clinical trials, 10 grams of LysPE38 has been produced by Yossi Shiloach and Amos Tsai, in the pilot plant at NIH. This material, plus 10 grams of clinical grade antibody were sent to Inland Laboratories where clinical grade, B3-LysPE38 (LMB-1) was prepared and sent to the NIH pharmacy and is currently available for a clinical trial (IND 5017). Toxicology studies carried out in collaboration with J. Tomaszewski, Division of Cancer Treatment, established that the maximum tolerated dose in monkeys is 4 mg/kilo. The clinical trial will begin at 0.1 mg/kilo given every other day for a total of 3 doses. The patients to be treated will have advanced cancers which react with MAb B3 and will have failed other therapies.

A single chain recombinant antibody containing the combining region of MAb B3 is B3(Fv)-PE38. L. Pai chose this one from a group of several molecules made by U. Brinkmann for clinical development. LMB-7 also causes complete regression of xenografts growing in nude mice. It has a molecular weight of 62,000 and its small size should enable it to penetrate into tumors better. It also is more active than LMB-1, it is more homogeneous, and requires fewer steps in its production. Monkey toxicity trials with LMB-1 are underway and a corporate partner is being sought for its production. It is estimated that 5 grams will be needed to carry out a complete Phase I study. One unexpected and potentially very useful finding is that LMB-7 does not cause liver toxicity in monkeys. This has been the dose limiting toxicity in mice.

A major problem in the immunotoxin field is the immunogenicity of the foreign proteins in man. This problem has been overcome with other types of proteins by attaching polyethylene glycol to the surface of these proteins. We have been examining the amino acids on the surface of PE38 to determine which amino acids can be derivatized with PEG without loss of activity. Several locations in domain II and domain III have been identified by Drs. Kuan and Benhar. Using these observations, Dr. Wang has developed chemical methods to carry out derivatization of PE mutants with polyethylene glycol. In addition, D. Roscoe has begun to map the major immunogenic sites on LMB-1. She is using a set of overlapping peptides that cover all of this molecule. She has also prepared several different mutant proteins in which regions of domains II and III are altered or deleted. She has used these reagents to determine major immunogenic epitopes on LMB-1.

Q.-C. Wang has made a modified form of TGF $\alpha$ -PE40 in which an antibody C<sub>H</sub>2 domain has been inserted between TGF $\alpha$  and PE40. Other lysine residues in this molecule have been mutated so that PEG can only react with the  $\alpha$  amino groups in the C<sub>H</sub>2 domain or at the N terminus of TGF $\alpha$ . This PEGylated molecule (TGF $\alpha$ -C<sub>H</sub>2-PE38QQR) retains biological activity, has an increased survival in the blood of mice, and diminished immunogenicity. This experiment supports the hypothesis that it should be possible to obtain active PEGylated immunotoxins. The methodology developed by Q.-C. Wang will be used for the B3 derived immunotoxins in combination with the results obtained by Drs. Roscoe, Benhar and Wang.

In collaboration with O. Gansow and J. Carrasquillo, L. Pai has made an indium and yttrium labeled chelate of monoclonal antibody B3. <sup>111</sup>Indium B3 images tumors in nude mice and has low uptake by liver. The usefulness of these agents for imaging and therapy in patients will be evaluated in a Phase I clinical trial in patients with metastatic carcinoma. The projected starting date is late 1993.

R. Kreitman has continued the analysis and development of anti-Tac(Fv) toxins. He has developed an animal model using A431 cells transfected with the p55 subunit of the IL2 receptor.

The cells (ATac-4) rapidly form tumors. He has shown that several different anti-Tac(Fv)-PE derivatives cause complete regressions of these tumors. He has also studied the pharmacokinetics of these molecules. For clinical development, we have chosen anti-Tac(Fv)-PE38 and as a back-up compound anti-Tac(Fv)-PE38KDEL. Monkey toxicity trials are underway to determine how much of this material can be given safely to monkeys. (Anti-Tac reacts equally well with humans and monkeys). R. Kreitman previously established that IL6-PE<sup>4e</sup> was an active agent against myeloma cells. In collaboration with Drs. Epstein and Barlogie, University of Arkansas, we are preparing drug for a bone marrow purging study for patients with multiple myeloma undergoing autologous bone marrow transportation. W. Debinski and R. Kreitman in collaboration with R. Puri, (FDA) have been studying a human IL4-toxin fusion protein. This agent is active against many cell lines that display human IL4 receptors and causes regressions of IL4 receptor bearing cancers. One of these, hIL4-PE38QQR, has caused complete regressions of IL4 receptor bearing A431 cells in mice. Because these recombinant molecules have weak binding to the IL4 receptor, we are exploring alternative ways of combining IL4 and the toxin to increase binding and thereby specific cytotoxic activity.

To make agents for the treatment of prostate cancer, we have developed a new antibody, termed MAb PR1, that reacts with a cell surface antigen present on normal prostate, BPH, and almost all prostate carcinomas. The antibody is an IgM. The variable regions of this antibody have been cloned by U. Brinkmann and used to make a single chain immunotoxin termed PR1(Fv)-PE38KDEL. This agent has specific cytotoxic activity towards cells bearing the PR1 antigen. However, there is no cell line which has good antigen expression. Therefore, evaluation of this molecule in a tumor model is not yet possible. We are trying to develop cell lines expressing the PR1 antigen by cloning the antigen and using the cDNA to construct such cell lines. U. Brinkmann has also inserted the PR1 variable regions into a mammalian expression vector containing the human constant region. Small amounts of antibody have been obtained which specifically bind to PR1 expressing cells. Our goal is now to scale up antibody production and possibly construct a fully humanized molecule. The PR1 antigen appears to be a protein since it is destroyed by protease and not by periodate. However, the identity of the antigen is not yet established.

TGF $\alpha$ -PE40 and derivatives of it have been shown to be active agents that cause regressions of subcutaneous tumors in mice bearing EGF receptors. In collaboration with Alan Oliff and Michael Goldberg at Merck, a Phase I clinical trial has been completed in which TP40 has been used in patients with superficial bladder carcinoma. In this trial involving patients who had failed many previous therapies, TP40 was shown to be safe up to a dose of 9.6 mg per dose when given into the bladder in a total volume of 60 mL once a week for 6 weeks. This is the same protocol as is used for BCG. Currently, plans for a Phase II trial are being developed. In an effort to make a more active drug, C. Theuer has constructed a new molecule in which proteolytic processing is no longer necessary. This compound termed PE35/TGF $\alpha$ -KDEL is 10- to 100-fold more active than TP40 on bladder cancer cell lines. He is setting up a model of bladder carcinoma in mice to compare the activity of these agents.

A. Kihara in collaboration with R. King at Molecular Oncology has been developing a recombinant immunotoxin targeted to cancers overexpressing the erbB2 protein. The agent selected for development of e23(Fv)-PE38KDEL. This agent causes regressions of tumors in nude mice. A. Kihara has made several mutant forms of this recombinant forms of this immunotoxin. One of these with a mutation at position 416 appears to be less toxic to animals and its activity against

tumors is being evaluated. Furthermore, the KDEL variant of this immunotoxin is about 10- to 20-fold more active on many human cancer cell lines. A. Kihara is exploring the basis of this unexpected increased activity.

C. Theuer and J. Buchner with D. FitzGerald have initiated studies on the mechanism of translocation of the 37 Kd fragment of PE. They have inserted a cecropin signal sequence prior to the 37 Kd fragment and using rabbit reticulocytes and pancreatic microsomes to insert the newly synthesized polypeptide into microsomes. They have found that amino acids 280 to 313 of PE prevent the molecule from being inserted into the ER. Furthermore, they can graft the 280-313 sequence onto prolactin and show that it also is not inserted into the ER. These findings are consistent with a model in which the 37 Kd fragment of PE (280-613) is translocated from the endoplasmic reticulum into the cytosol through channels in the ER. These channels are the ones used for the secretion of proteins into the ER from the cytosol. Work by D. FitzGerald and colleagues has shown that other sequences in domain II are probably required to direct the 37 kilodalton fragment from the endocytic compartment through the trans Golgi into the endoplasmic reticulum.

E. Mesri and R. Kreitman have developed chimeric toxins in which a heparin binding domain in association with either TGF $\alpha$  or EGF directs *Pseudomonas* toxin to target cells. These agents have high cytotoxicity towards cells expressing the heparin binding EGF receptor and are useful in establishing the interactions of heparin binding EGF with receptors on target cells.

K. Webber has initiated studies on single chain antibodies made with K1 and B3 and will compare their binding activities with that of Fab fragments made from the same molecules. These agents should be useful in the imaging of tumors and also may be useful as therapeutic agents when coupled to radioisotopes.

#### CRADAs:

Contractor: MOLECULAR ONCOLOGY

CRADA Number: CACR 0122

Date Contract Initiated: 11/14/91

Summary: Several single chain immunotoxins were constructed containing the variable region of the e23 MAb and recombinant forms of PE. One of these e23(Fv)-PE38KDEL was chosen for clinical development. Preclinical studies are now being conducted in relevant animal models.

Contractor: HOFFMANN-LA ROCHE

CRADA Number: CACR 0065

Date Contract Initiated: 09/14/89

Summary: This project was directed at making a single chain immunotoxin that kills cells with IL2 receptors. The immunotoxin with the best ratio of efficacy to toxicity is anti-Tac(Fv)-PE38. This molecule is in preclinical development.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08754-10 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
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)		
PI:	I. Pastan	Chief, Laboratory of Molecular Biology NCI
Co-PI:	M. M. Gottesman	Chief, Laboratory of Cell Biology NCI
COOPERATING UNITS (If any) A. Nienhuis, Chief, Clinical Hematology Branch, NHLBI, MH		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Molecular Biology Section		
INSTITUTE AND LOCATION National Cancer Institute		
TOTAL STAFF YEARS: 13.5	PROFESSIONAL: 11.5	OTHER: 2.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <span style="float: right;">B</span>		
SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.) <p>We have continued to analyze the mechanism of action of the multidrug transporter and have worked on the development of new strategies to circumvent multidrug resistance in cancer and to exploit molecular knowledge of the multidrug transporter to design new cancer treatments. The multidrug transporter (P-glycoprotein) has been purified to near homogeneity and shown to be an active drug-dependent ATPase of high specific activity after reconstitution into proteoliposomes. Vesicles containing P-glycoprotein capable of transport have very active P-glycoprotein kinases, and this activity is stimulated by GTP. At least one novel plasma membrane associated P-glycoprotein-kinase has been partially purified, but its role in regulating activity of the multidrug transporter has not yet been determined. Kinetic studies demonstrate that the transporter interacts with drugs within the lipid bilayer, and indirect evidence suggests that drug may be removed from both the inner and outer leaflets of the bilayer. Molecular manipulation of P-glycoprotein by analysis of point mutations and chimeras with other members of the ATP-binding cassette (ABC) superfamily of transporters has revealed multiple regions of the molecule near or within the transmembrane domains which affect substrate specificity, and has indicated the interchangeability of ABCs between <i>MDR1</i> and <i>MDR2</i>, a related transporter of unknown specificity. Function of P-glycoprotein has been explored by insertional inactivation of the <i>mdr1b</i> gene in mouse adrenal Y-1 cells, with loss of ability of these cells to secrete steroids above basal levels. We have continued to develop the <i>MDR1</i> gene as a dominant selectable marker for gene therapy. Retroviral vectors expressing the human <i>MDR1</i> cDNA are able to confer resistance to taxol on transduced and transplanted mouse bone marrow cells, and this strategy is under consideration for gene therapy in humans to protect bone marrow during high dose chemotherapy for cancer. Two other multidrug resistant genetic systems are under development to aid in the analysis of other mechanisms of multidrug resistance: (1) A human melanoma line cross-resistant to epipodophyllotoxins (VP-16 and VM-26) and anthracyclines which has a deletion of Ala 428 in topoisomerase II; and (2) High level <i>cis</i>-platinum resistant human hepatoma and KB adenocarcinoma cells with multiple protein alterations.</p>		

Other Professional Personnel:

Y. Sugimoto	Special Volunteer	LCB, NCI
S. Zhang	Visiting Fellow	LCB, NCI
S. Ambudkar	Guest Researcher	LCB, NCI
B. Ni	Visiting Fellow	LCB, NCI
S. Goldenberg	Research Biologist	LCB, NCI
U. Germann	Visiting Associate	LCB, NCI
P. Schoenlein	Special Volunteer	LCB, NCI
D.-W. Shen	Visiting Associate	LCB, NCI
I. Aksentijevich	Senior Staff Fellow	LCB, NCI
F. Hsieh	Guest Researcher	LCB, NCI
C. Cardarelli	Research Biologist	LMB, NCI
J. Campaign	Special Volunteer	LMB, NCI
G. Evans	Biotechnology Fellow	LMB, NCI
M. Siegsmond	Special Volunteer	LMB, NCI
J. Aran	Special Volunteer	LMB, NCI
T. Licht	Special Volunteer	LMB, NCI

Major Findings:

1. We have continued to purify P-glycoprotein from plasma membrane vesicles of human KB-V1 multidrug resistant cells and from insect Sf9 cells infected with an *MDR1* baculovirus. Both are rich sources of active P-glycoprotein capable of drug-dependent ATPase activity. The KB-V1-derived P-glycoprotein is approximately 95% pure after octyl glucoside extraction and fractionation on DEAE Sepharose CL6-B cellulose and wheat germ agglutinin columns. There is a low level of basal ATPase activity of these preparations in detergent. After reconstitution into liposomes prepared from *E. coli* lipids, phosphatidyl choline, phosphatidyl serine and cholesterol, this activity can be stimulated several-fold by drugs and is vanadate-inhibitible. Specific activities of pure P-glycoprotein of 15-25 umole of phosphate hydrolyzed per mg protein have been calculated. This specific activity is comparable to that of Na-K ATPase. This drug-stimulation appears to be specific for known substrates of P-glycoprotein such as verapamil and vinblastine, but other hydrophobic drugs which are not transported, such as camptothecin, do not stimulate the ATPase activity. The purified, reconstituted protein is photoaffinity labeled specifically by azidopine and forskolin. The purified material with a molecular weight of 160-170 kDa in detergent, copurifies with two P-glycoprotein fragments of 110 kDa and 55 kDa which represent the amino glycosylated half and the carboxy terminal half of P-glycoprotein which appear to be tightly non-covalently associated with each other during purification.

2. Because of published reports suggesting that P-glycoprotein may be regulated by phosphorylation, we have studied the phosphorylation of P-glycoprotein in transporting vesicles prepared from KB-V1 cells. These vesicles contain one or more protein kinases capable of phosphorylating P-glycoprotein in the presence of ATP or GTP. GTP dramatically stimulates phosphorylation by ATP in this crude vesicle system by an unknown mechanism. This phosphorylation is completely inhibited by staurosporine, but not by traditional inhibitors of protein kinase A or C, suggesting that it is due to a novel kinase or kinases. We have begun to

purify one of the kinases from this vesicle system which is capable of phosphorylating purified P-glycoprotein preparations which are otherwise devoid of kinase activity. Preliminary results indicate that this partially purified membrane-associated kinase activity is not Ca-stimulated, but is inhibited by staurosporine, suggesting that it is a novel kinase which may play a significant role in physiological phosphorylation of P-glycoprotein.

3. In addition to these biochemical studies, we have continued a kinetic analysis of the uptake and efflux of drugs from multidrug resistant cells. These studies have been conducted on NIH 3T3 cells transfected with either a wild-type (G185) or mutant (V185) multidrug transporter which are expressed at approximately equal levels on the surface of these cells. Studies on the wild-type transporter indicate that hydrophobic drugs such as vinblastine, which are excellent substrates for the transporter, show decreased uptake from the earliest time points as well as increased efflux under conditions in which pump function is inhibited by energy deprivation to load the cells, and then re-energized. These studies support our model of P-glycoprotein as a "hydrophobic vacuum cleaner" capable of detection and ejection of drugs as they cross the plasma membrane, before they enter the cytoplasm. Interestingly, colchicine, a non-charged poorer substrate of P-glycoprotein, appears to be primarily effluxed by the pump, with little kinetic evidence of decreased uptake. The mutant transporter, in contrast, shows both decreased uptake and increased efflux of colchicine, but the initial rate of vinblastine uptake is higher than for the wild-type, consistent with the increased efficiency of transport of colchicine and decreased efficiency of vinblastine transport by the mutant. These results argue that there may be at least two components to detection of drugs in the membrane, consistent with removal from the inner or outer leaflets of the plasma membrane, or to multiple entry points along the transporter for drugs. In addition, inhibitors interact differently with mutant and wild-type transporters. For example, cyclosporine A very efficiently inhibits the mutant transporter compared to the wild-type, and verapamil is a more efficient inhibitor of the wild-type transporter.

4. Molecular genetic approaches have also been taken to study the mechanism of action of P-glycoprotein. Several chimeras and mutants have been shown to have altered function. A chimera formed when the first intracytoplasmic loop of *MDR2* (non-functional in drug transport) is substituted into the *MDR1* transporter results in loss of function, which can be returned by replacement of 4 out of 13 of the altered amino acids. This result argues that the *MDR2* transporter is not that different from *MDR1*, at least in this region. Chimeras in which different ATP-binding cassettes (ABCs) from *MDR2* and the cystic fibrosis transmembrane regulator (*CFTR*) have been substituted into *MDR1* suggest that *MDR2* has active ABCs, but that ABCs from *CFTR* are non-functional in *MDR1*. A deletion mutant lacking the amino acids encoded by exon 20 (residues 800-827) which removes part of the putative fourth intracytoplasmic loop is fully functional for transport of anthracycline and taxol, but has reduced transport of actinomycin D and Vinca alkaloids. These results argue that it should be possible to design multidrug transporters with specific alterations in drug specificity, and that some internal deletions in P-glycoprotein can be tolerated without major loss of function. They also suggest a certain compartmentalization of P-glycoprotein activities which affect substrate specificity, consistent with more than one site of drug-protein interaction.

5. To facilitate additional manipulations of the transporter for studies of mechanism and to design transporters with altered substrate and inhibitor specificity for gene therapy (see below), we have explored the use of model organisms for functional expression of P-glycoprotein. Studies in

which human P-glycoprotein is expressed under control of the lambda P<sub>L</sub> promoter indicate that expression of the full-length *MDR1* cDNA is extremely toxic to *E. coli*, with induction of expression resulting in a rapid drop in viability and cessation of P-glycoprotein protein synthesis. Expression in *Saccharomyces cerevisiae* is, however, possible with at least two promoter-plasmid systems. P-glycoprotein in yeast membrane preparations is easily detected on Western blots, and binds azidopine in a specific manner. When P-glycoprotein is expressed in yeast strains sensitive to hydrophobic cytotoxic drugs, resistance to valinomycin, daunorubicin, and actinomycin D can be demonstrated. These studies suggest that yeast may be a suitable organism for molecular structure-function studies of P-glycoprotein.

6. Multidrug resistance encoded by the *MDR1* gene is a dominant selectable marker suitable for use *in vivo* as well as *in vitro*. Retroviral vectors using either a Harvey sarcoma virus promoter, or a Moloney promoter, have been constructed and a variety of producer cell lines (GP+E86, AM12) have been selected which have high supernatant titers of transducing *MDR1* retrovirus (>1 x 10<sup>9</sup>/ml). These supernatants are free of non-defective virus and are therefore suitable for gene therapy experiments. Mouse bone marrow transduced with these retroviruses can be transplanted into host animals and continues to express the *MDR1* gene several months after transplant. After selection *in vivo* with taxol, *MDR1*-containing marrow cells become more prominent in the peripheral circulation, suggesting that the *MDR1* gene has conferred a selective advantage on the transduced marrow. The finding of long-term transplantable *MDR1*-expressing cells, and evidence for similar DNA insertion sites in peripheral cells of different lineages (lymphocytes and granulocytes), indicates that stem cell transduction has occurred in the mouse. Previous evidence from our *MDR1* transgenic mice, and from transduction of erythroleukemia cells suggests that relatively high level expression of the *MDR1* gene in bone marrow has little or no deleterious effect on function. These experiments, combined with the ease of selection of the multidrug resistant phenotype, cell surface expression of P-glycoprotein which allows for detection and sorting of living cells, and the potential ability to "design" unique transporters functionally distinguishable from the wild-type transporter, indicate that the *MDR1* gene is a good candidate for gene therapy in humans. Since protection of human bone marrow from the toxicity of anti-cancer therapy using the *MDR1* gene should be possible, clinical trials testing the feasibility of this approach in patients with breast and ovarian cancer undergoing autologous bone marrow transplantation are under consideration.

7. The ability of the *MDR1* gene to act as a selectable marker in gene therapy suggests that it could be used to select for the transfer of other genes into cells *in vivo*. We are currently designing vectors in which expression of non-selectable genes should occur after selection for multidrug resistance. Two approaches have been taken: (1) production of chimeric proteins, in which non-selectable genes are fused to the 3' end of the *MDR1* cDNA to produce bifunctional molecules. This approach has been previously shown to work with adenosine deaminase, which is fully functional when fused to P-glycoprotein; and (2) production of polycistronic mRNAs in which both P-glycoprotein and a second protein are separately encoded each with its own translation initiation region, separated by an internal ribosomal entry site derived from encephalomyocarditis virus. A variety of cDNAs including those encoding proteins defective in lysosomal storage diseases are being utilized for these studies.

8. To study the normal role of P-glycoprotein in transport of endogenous compounds, we have inactivated an *mdr* gene in cultured mouse adrenocortical Y1 cells. These cells synthesize and secrete steroids in large amounts, and they contain highest amounts of the *mdr1b* gene, with only small amounts of *mdr1a* and *mdr2* mRNA. We have prepared a homologous recombination vector with a G418 resistance cassette inserted into the *mdr1b* gene as a positive selection and one HSV-TK gene at each end of the *mdr1b* sequences to use for negative selection against non-homologous inserts. Approximately 1 of 150 G418 resistant Y1 clones had inserted the G418 resistance cassette into the center of the *mdr1b* gene, thereby inactivating one of two *mdr1b* alleles in this cell line. This insertion resulted in the inability of the mutant Y1 cells to secrete steroids above basal levels in response to ACTH. Paradoxically, the *mdr1b* mRNA from the second allele increased in amount approximately 10-fold, suggesting that this second allele encoded a functionally inactive transporter which was up-regulated by a feedback mechanism. These results suggest that P-glycoprotein may play an important role in secretion of steroids above basal levels.

9. We have selected human melanoma cells in the presence of VP-16 (etoposide) and a potent inhibitor of the multidrug transporter. We obtained mutants cross-resistant to the non-intercalative topoisomerase inhibitors VP-16 and VM-26, with lower levels of resistance to anthracyclines. As expected, resistant mutants do not express P-glycoprotein, but one series of mutant cell lines of increasing resistance express a mutant DNA topoisomerase II $\alpha$ . The mutation deletes amino acid 428 (an alanine). With increasing levels of resistance to VP-16, the relative percentage of mRNA encoding the mutant topoisomerase increases, although the total amount of mRNA and protein does not change, suggesting that this mutation is responsible for the resistance to VP-16 and VM-26. Because this mutation is in a somewhat different region of topoisomerase II from other recently described mutants, and because nuclear extracts contain normal amounts and activity of topoisomerase II, we hypothesize that this mutation may affect an interaction of topoisomerase II with another component of the nucleus needed for sensitivity to anti-cancer drugs.

10. Mutants of human KB adenocarcinoma and human hepatoma cells resistant to high levels of *cis*-platinum have been isolated in multiple steps. Two dimensional gel electrophoresis of extracts of these cells show a large number of changes in proteins, but an increase in amount of a protein(s) of approximate molecular weight 52 kDa is consistently seen in both cell types. Expression of the 52 kDa protein(s) is stably increased in the absence of *cis*-platinum, and revertant cells which have lost *cis*-platinum resistance have reduced amounts of the protein(s). To characterize the genetic changes in the *cis*-platinum resistant hepatoma cells, we have transferred DNA from these cells to drug sensitive mouse fibroblasts and obtained *cis*-platinum resistant primary and secondary transfectants, with human Alu sequences linked to transfer of the resistance phenotype. Efforts to clone the genomic DNA associated with transfer of *cis*-platinum resistance are underway.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08756-06 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Transgenic Mouse as a Model System to Study Gene Function and Regulation		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute)		
PI:	G. T. Merlino	Senior Investigator LMB, NCI
Others:	M. M. Gottesman	Chief, Laboratory of Cell Biology NCI
	C. Jhappan	Senior Staff Fellow LMB, NCI
	H. Takayama	Visiting Fellow LMB, NCI
	J. Jakubczak	IRTA Fellow LMB, NCI
	R. Sharp	Biochemist LMB, NCI
COOPERATING UNITS (If any) N. Fausto, Laboratory of Pathology & Laboratory Medicine, Brown University; A. Roberts, Laboratory of Chemoprevention, NCI; G. Smith, Laboratory of Tumor Immunology and Biology, DCBDC, NCI; Snorri Thorgeirsson, Laboratory of Experimental Carcinogenesis, NCI		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Molecular Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS: 5.0	PROFESSIONAL: 4.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <span style="float: right;">B</span>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Transgenic technology, in which foreign DNA is stably introduced into the mammalian gene line, represents a method to address basic biological questions that is both powerful and versatile. We are using this exciting technology to examine the role of growth factors, receptors and oncogenes in tumorigenesis, and to establish useful and novel animal models to aid in the study of pathogenesis in human disease.</p> <p>Transforming growth factor alpha (TGF <math>\alpha</math>) stimulates cellular proliferation by binding to the epidermal growth factor (EGF) receptor and activating its tyrosine kinase. Perturbation of this signal transduction pathway can transform cells in culture, and has been implicated in human oncogenesis. To test this hypothesis <i>in vivo</i>, mice were made harboring a human TGF<math>\alpha</math> transgene. Overexpression of TGF<math>\alpha</math> was found to induce hepatocellular carcinoma, mammary adenocarcinoma, pancreatic metaplasia and fibrosis, and a hypertrophic gastropathy resembling Menetrier's disease. Detailed molecular analysis of lesions in these mice has confirmed that TGF<math>\alpha</math> promotes tumor formation and plays a role in tumor progression. Analysis of double transgenic mice demonstrated that TGF<math>\alpha</math> and the <i>c-myc</i> nuclear protooncogene act in a synergistic fashion in hepatocarcinogenesis. Furthermore, TGF<math>\alpha</math> was able to collaborate with diverse chemical agents in the development of liver tumors, including genotoxic initiators and nongenotoxic promoters.</p> <p>We have generated mice bearing transgenes encoding other relevant growth and differentiation factors. Transgenic mice made with an activated form of an EGF-related gene, <i>int-3</i>, which contains numerous EGF repeat-sequences and is a member of the <i>Notch</i> gene family, develop severe hyperplastic and developmental lesions of multiple secretory glands and cancer of the salivary and mammary glands. These findings demonstrate <i>in vivo</i> that expression of the activated <i>int-3</i> gene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelia. In another study, mice overexpressing a transforming growth factor <math>\beta</math>1 (TGF<math>\beta</math>1) transgene in the pregnant mammary gland were unable to lactate due to the inhibition of the formation of lobuloalveolar structures and suppression of endogenous milk production. These results strongly suggest that TGF<math>\beta</math>1 plays an important role in regulating the development and function of the mammary gland.</p>		

Major Findings:

Transgenic mice are generated by microinjecting purified DNA fragments into the pronuclei of single-cell mouse embryos derived from the FVB/N inbred strains of mouse. Microinjected embryos are transferred into pseudopregnant CD-1 foster mothers. Transgenic pups are identified by Southern Blot and/or polymerase chain reaction analysis of genomic DNA isolated from tail biopsies. Colonies of mice heterozygous for a transgene are established, and when appropriate, heterozygotes are mated to produce homozygous animals. Transgene expression is determined by Northern blot and RNase protection analysis of total RNAs from a variety of tissues.

Overexpression of the EGF receptor and its ligands (TGF $\alpha$  and EGF) can transform cells in culture. To determine the *in vivo* consequences of perturbing the EGF receptor signal transduction pathway, we produced mice bearing TGF $\alpha$  and/or EGF receptor transgenes. In one series of experiments, a DNA fragment containing a human TGF $\alpha$  cDNA driven by the mouse metallothionein-1 promoter was microinjected into mouse embryos. Mice bearing this transgene expressed abundant TGF $\alpha$  RNA and protein in multiple tissue types, including the liver, pancreas, stomach and breast. Elevated levels of TGF $\alpha$  were detected in the blood and urine of transgenic mice, as has been shown in cancer patients. TGF $\alpha$  transgenic mice developed many dramatic lesions, making them useful as animal models for a number of important human diseases.

One year old male transgenic mice exhibited a high incidence (about 75%) of liver tumors, most of which greatly overexpressed the TGF $\alpha$  transgene. To identify collaborating factors associated with TGF $\alpha$  induced oncogenesis, liver tumors from transgenic animals were characterized at the molecular level. Expression of the endogenous *c-myc* and insulin-like growth factor II genes was enhanced frequently in liver tumors. The ability of *c-myc* to cooperate with TGF $\alpha$  in liver tumor development was tested further by mating our TGF $\alpha$  transgenic mice with *c-myc* transgenic mice (made in the laboratory of Dr. Snorri Thorgeirsson) in which the *c-myc* proto-oncogene was overexpressed in the liver. Severe dysplastic lesions were observed in the livers of double transgenic mice by 6 weeks of age, and frank liver tumors appeared as early as 3 months of age. This result illustrates the power of the transgenic mouse technology, and confirms that potent growth factors and nuclear protooncogenes can collaborate in hepatocarcinogenesis.

To determine more precisely how TGF $\alpha$  participates in liver tumorigenesis, initiating genotoxic and promoting nongenotoxic chemical carcinogens were administered independently to TGF $\alpha$  transgenic male mice. Both types of agents were found to enhance strongly both hepatic tumor formation and progression. These results show that diverse chemical agents can act as cocarcinogens with TGF $\alpha$  in the transgenic livers, and indicate that TGF $\alpha$  possesses the unique ability to complement both initiation and promotion in hepatocarcinogenesis. Interestingly, these agents did not affect hepatic tumorigenesis in female TGF $\alpha$  transgenic mice, underscoring the role of sex hormones in this process.

TGF $\alpha$  transgenic mice also developed mammary adenomas and adenocarcinomas in about half of all multiparous female mice by about nine months of age. Interestingly, tumors were quite rare in virgin animals, suggesting a strong role for pregnancy-associated hormones in TGF $\alpha$ -induced breast cancer. To examine early stages in mammary oncogenesis, hyperplastic alveolar nodules were removed from young, parous transgenic females, and transplanted into syngeneic recipient mammary fat pads (in collaboration with Dr. Gilbert Smith). These hyperplastic lesions did not persist through multiple transplantations, and rarely developed into tumors, suggesting that TGF $\alpha$  overexpression alone was not sufficient to immortalize mammary epithelial cells. In contrast, transplanted tumor cells from these transgenic mammary glands were able to develop into malignant lesions.

TGF $\alpha$  also induced dramatic structural and functional lesions of the glandular stomach that resemble Menetrier's disease in humans. Transgenic mice developed in an age-dependent fashion severe adenomatous hyperplasia that resulted in a striking nodular thickening of the gastric mucosa. Secretions obtained from affected stomachs contained no detectable gastric acid, suggesting that the parietal cell population had been compromised. This was confirmed by quantifying levels of RNA encoding a parietal cell-specific ATPase, which was greatly reduced in older transgenic stomachs. These findings demonstrate that, when inappropriately expressed, TGF $\alpha$  can stimulate cellular proliferation, suppress acid secretion and perturb organogenesis of the stomach. Moreover, TGF $\alpha$  may contribute to the pathogenesis of related human stomach disorders such as Menetrier's disease.

Finally, TGF $\alpha$  induced a florid ductular metaplasia of the exocrine pancreas, and severe interstitial fibrosis in these transgenic animals. The pancreas was greatly enlarged due to an increase in connective tissue. Acinar cells appeared to redifferentiate into ductular cells and mucin-secreting cells, forming tubular complexes. The transgenic pancreas routinely displayed properties that are characteristic of diseases of the human pancreas. Therefore, the TGF $\alpha$  transgenic mouse may represent a valuable animal model for the study of the development and treatment of human pancreatic disease. Furthermore, changes in the relative numbers of various differentiated cell types in both the pancreas and stomach suggest a role for TGF $\alpha$  in cellular differentiation.

In another series of experiments, transgenic mice were made bearing a foreign DNA fragment containing a human EGF receptor cDNA. In one unique line of mice pronounced expression of the human EGF receptor was detected only in the testis. Homozygous male mice were sterile due to axonemal aberrations resulting in sperm paralysis. Axonemal aberrations of this type have been observed in the sperm of sterile men. This unique transgenic mouse line should provide a useful model for studying male infertility.

We are also using transgenic mice to study growth factors and receptors that are related to EGF. The gene encoding one such peptide (*int-3*) was identified by its role in mouse mammary gland oncogenesis. The mouse mammary tumor virus (MMTV) has been shown to integrate into, and activate the *int-3* gene, which contains sequence repeats similar to the yeast *cdc10* cell cycle "start" gene and encodes multiple EGF repeats. We recreated this integration event in a system amenable to detailed analysis by generating transgenic mice harboring a DNA fragment containing the activated *int-3* gene under the transcriptional control of the MMTV long terminal repeat.

All six *int-3* founder transgenic mice and all the members of one established line exhibited similar dramatic phenotypic abnormalities in all tissues in which the transgene was expressed. Focal and multiple poorly differentiated mammary and salivary adenocarcinomas appeared in the majority of transgenic mice between two and seven months of age. Mammary glands were arrested in development and lactation-deficient in all female *int-3* mice. Hyperplastic immature ductule cells appearing to be incompletely differentiated were observed in all salivary glands, and in numerous glands of the head and neck of juvenile and adult transgenic mice. In addition, all male transgenic founders were sterile due to severe hyperplasia of the epididymis. These findings demonstrate *in vivo* that expression of the activated *int-3* gene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelial. In addition, we have produced double transgenic mice containing both the TGF $\alpha$  and *int-3* transgenes. These animals exhibited highly accelerated mammary gland tumorigenesis, suggesting that these two factors can function synergistically in the development of breast cancer.

Transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) possesses highly potent and diverse cell-specific activities. These can either complement or oppose proliferative activities associated with TGF $\alpha$ . To determine

the effects of *in vivo* overexpression of TGF $\beta$ 1 alone, and to elucidate interactive mechanisms between TGF $\beta$ 1 and TGF $\alpha$ , we have made and analyzed transgenic mice expressing a TGF $\beta$ 1 gene in the pregnant mammary gland by virtue of the whey-acidic protein gene promoter. Female transgenic mice overexpressing TGF $\beta$ 1 in the mammary gland were unable to lactate due to inhibition of lobuloalveolar structure formation and suppression of endogenous milk production, supporting an *in vivo* role for TGF $\beta$ 1 in regulating the development and function of the mammary gland. To determine if TGF $\beta$ 1 can suppress the development of breast cancer, we are presently generating female mice that are transgenic for both TGF $\beta$ 1 and TGF $\alpha$ .

To determine the transcriptional mechanisms by which proto-oncogenes are regulated, we had previously isolated and characterized the EGF receptor gene promoter. To study *in vivo* promoter function, fragments of genomic DNA containing all known EGF receptor gene *cis* elements required for optimal activity in transfected cultured cells were used to drive the expression of the chloramphenicol acetyltransferase reporter gene in transgenic mice. Unexpectedly, in all expressing lines of transgenic mice the EGF receptor gene promoter was active primarily in the thymus and secondarily in the spleen. Cell separation techniques showed that the promoter was not active in the thymocytes, but only in the stromal tissue, containing thymic epithelial cells. Our results raise the possibility the EGF receptor may play some role in thymic epithelial cell function and/or thymocyte selection. We believe that this novel promoter will be useful in the future to target expression of various transgenes to the thymic epithelium.

In another project, we are continuing to collaborate with Drs. Pastan and Gottesman in the generation and analysis of transgenic mice containing a human gene (*MDR1*) encoding the 170 kDa P-glycoprotein. P-glycoprotein is capable of conferring multidrug resistance to animal cells, and when overexpressed allows malignant cells to evade destruction by chemotherapeutic agents. In our initial efforts, transgenic mice were generated in which a human *MDR1* cDNA was expressed in the transgenic bone marrow, which in nontransgenic animals is exquisitely sensitive to chemotherapy. In these animals it was determined that the transgenic bone marrow was resistant to drug-induced leukopenia, and that bone marrow suppression was either greatly ameliorated or eliminated. Furthermore, drug resistance in these transgenic mice could be overcome by the administration of appropriate reversing agents, such as verapamil. Ultimately, we plan to use multiple lines of transgenic mice expressing the *MDR1* transgene in various subsets of blood cells as *in vivo* model systems to test the efficacy of novel chemotherapeutic drugs or combinations of drugs, including reversing agents which inhibit activity of the multidrug transporter.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08757-06 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
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)		
PI:	D. J. FitzGerald	Microbiologist LMB, NCI
Others:	I. Pastan	Chief, Laboratory of Molecular Biology NCI
	M. Chiron	Visiting Fellow LMB, NCI
	A. Zdanovsky	Visiting Associate LMB, NCI
	C. Fryling	Biologist LMB, NCI
	M. Zdanovskaiva	Special Volunteer LMB, NCI
COOPERATING UNITS (if any) C. B. Saelinger, Dept. of Microbiology and Molecular Genetics, University of Cincinnati Medical School, Cincinnati, OH; D. Strickland, American Red Cross, Rockville, MD		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Ultrastructural Cytochemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.2	4.2	1.0
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		B
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Interactions of <i>Pseudomonas</i> exotoxin (PE) with mammalian cells were studied. PE binds to cells via the $\alpha 2$ -macroglobulin receptor ( $\alpha 2$ -MR). PE but not PEglu57 bound to affinity purified $\alpha 2$ -MR. The addition of receptor associated protein (RAP) blocked PE-mediated toxicity but did so without competing for the same binding site. Binding to the $\alpha 2$ -M receptor on cells is followed by endocytosis. Within cells PE is cleaved between arg279 and gly280 to produce an N-terminal fragment of 28 kD and a C-terminal fragment of 37 kD. A protease with this activity was prepared from beef liver (BLP). In biochemical assays, the cleavage of PE by the BLP was optimal at pH 5.5, was inhibited by EDTA or p-hydroxymercuribenzoate and had a Km of approx 1.0 mM. When PE was cleaved by the BLP and then added to cells, cleaved toxin killed cells with greater rapidity than native PE. Cleavage of PE by BLP resembled the activity of a furin-like protease. To confirm this, PE was incubated with recombinant human furin. Furin-mediated cleavage of PE was indistinguishable from the activity seen with the BLP. The beef liver protease and furin also cleaved diphtheria toxin. In separate experiments, a rate-limiting step in toxin action was discovered. After cleavage of PE by cells, residues at the N-terminus of the 37 kD C-terminal fragment were shown to interact with an unknown cellular component in a saturable manner. To demonstrate this, excess PE $\Delta$ 553 was shown to compete for the toxicity of native PE. In contrast, the addition of excess PE harboring mutations at residues 281, 284 or 289 competed much less well. Competition, which was not at the level of surface binding, required intracellular toxin cleavage but did not require the presence of REDLK at the C-terminus.		



### Major Findings:

Characterization of PE binding to the  $\alpha_2$ -macroglobulin receptor.

*Pseudomonas* exotoxin (PE) kills multiple cell types from many different species. To be broadly effective, PE must interact with molecules that are highly conserved in nature. The  $\alpha_2$ -macroglobulin receptor ( $\alpha_2$ -MR), which serves as the cell surface receptor for PE, is a large cell-surface glycoprotein composed of polypeptides of 515 kDa and an 85 kDa. This receptor is widely distributed on different tissues, is highly conserved between species and is expressed at high levels on hepatocytes and fibroblasts. The receptor itself is synthesized as a 600 kD precursor and is proteolytically processed within cells to generate an N-terminal 515 kD alpha chain which is displayed at the cell exterior and a C-terminal 85 kD beta chain which has a transmembrane sequence and has residues displayed both at the cell exterior and cytoplasmically. The two chains are linked by a non-covalent attachment. Many ligands bind to the receptor's alpha chain. These include activated  $\alpha_2$ -macroglobulin, apoE-enriched  $\beta$ -VLDL, receptor-associated protein (RAP), PAI-1 when complexed with plasminogen activators, pregnancy zone protein, lipoprotein lipase and *Pseudomonas* exotoxin (PE). Three of these ligands, RAP, PAI-1 and PE, bind to immobilized receptor after it has been resolved by SDS-PAGE and electroblotted to nitrocellulose. Therefore, it may be possible to locate ligand binding sites by expressing receptor fragments, transferring them to nitrocellulose and probing for ligand binding.

To accomplish this, 13 separate clones corresponding to the entire alpha chain and a small portion of the beta chain were generated by PCR (Fig 1). Individual clones were expressed in *E. coli* and analyzed by SDS-PAGE. Resolved proteins were also electroblotted to nitrocellulose membranes and probed with a rabbit antiserum that had been raised against human  $\alpha_2$ -MR. Results indicated that receptor fragments were expressed at variable levels and that fragments 3, 9 and 13 were recognized most strongly by the anti-receptor antibodies. When ligand blots were performed there was no evidence of specific binding to any of the 13 expressed clones. Therefore combinations of clones were joined to each other and expressed as larger recombinant proteins. Results indicated that again there was variable expression of receptor protein and variable reactivity with the rabbit anti-receptor antisera.

Previously it was shown that the addition of RAP to cells blocked PE-mediated toxicity. To investigate the mechanism for this inhibition, several approaches were taken. To determine if RAP was a competitive inhibitor for PE binding, ligand blots were performed on immobilized receptor. When the receptor was incubated with 2  $\mu$ g/ml of PE and then probed with RAP, there was no decrease in RAP binding. When the reciprocal experiment was performed, there was a slight (no more than 2-fold) reduction in PE binding when 2  $\mu$ g/ml of RAP was added to receptor. The result suggested that RAP and PE did not compete for the same binding site on the receptor. The possibility that RAP was acting as a non-competitive inhibitor was also considered. The was addressed indirectly. If RAP bound to the  $\alpha_2$ -MR and was subsequently internalized it should be able to transport the toxic portion of a toxin to the endocytic compartment. A chimeric toxin between DT and RAP was constructed and had poor cytotoxic activity for cells expressing the  $\alpha_2$ -MR. This suggested that RAP binding did not lead to rapid internalization to the endocytic compartment and in fact may inhibit the internalization of this receptor.

Identification and biochemical characterization of the furin-like endopeptidase that processes *Pseudomonas* exotoxin.

Once inside cells, PE is cleaved between Arg279 and Gly280 (Ogata et al, J Biol Chem 1992;267:25396-401) by a cellular protease and then reduced to generate two prominent fragments, (Ogata et al, J Biol Chem 1990;265:20678-85). The N-terminal fragment is 28 kD in size and contains the binding domain. The 37 kD C-terminal fragment contains the translocation domain and the ADP-ribosylation domain and is responsible for causing cell death. Cleavage of PE is essential for toxicity since mutant forms of the toxin that cannot be cleaved are non toxic.

To characterize the protease responsible for cleavage, a protease-enriched preparation was prepared from beef liver. Instead of PE, which is a poor substrate, purification was monitored using PEala281 as the substrate. A furin-like protease that cleaves PE between Arg279 and Gly280 was purified approximately 500-fold from beef liver. After tissue homogenization, the relevant proteolytic activity was released from liver membranes by limited digestion with papain. The released protease was purified 100-fold in several steps: dialysis against 20 mM Na acetate at pH 5.5 was followed by chromatography on Blue Sepharose, QM resin, Arginine Sepharose and DEAE nonporous resin. When PE was used as the substrate, the protease generated an N-terminal fragment of 28 kD and a C-terminal fragment of 37 kD. Proteolysis had a pH optimum of 5.5, was inhibited by EDTA or p-hydroxymercuribenzoate but not by O-phenanthroline, N-ethylmaleimide, E-64 or PMSF (or other well known inhibitors of serine proteases). Under apparently optimal conditions, the Km for native PE was estimated to be 1-3  $\mu$ M. However very little product was generated. When PE was cleaved by the BLP and added to cells, the rate of inhibition of protein synthesis exceeded the rate by native PE. The level of inhibition by cleaved PE at four hours post-toxin addition was comparable to that of uncleaved PE at six hours post-toxin addition. Other proteins were tested as substrates. A mutant form of PE, PEala281, was cleaved at the same Arg-Gly site as native PE, with the same pH optimum and same Km but the production of the toxin fragments occurred with much greater efficiency. Un-nicked diphtheria toxin was cleaved at high efficiency. Cleavage was between the A and B chains on the C-terminal side of the sequence RXRR (aa-190-193). The Km for DT as a substrate has not yet been established but cleavage was seen at pH values from 5.5 -8.5, with the optimum at pH8.0.

The requirement for a divalent cation, the pattern of inhibitor action and the presence of arginine residues at the -1 and -4 positions relative to the cleavage site, suggested that the BLP was a furin-like enzyme. Furin is a member of the mammalian subtilisin family. Mammalian subtilisins are responsible for the maturation of many secreted proteins and, in neuroendocrine tissue, for the processing and release of peptide hormones from their inactive precursors. These subtilisins are serine proteases that cleave substrates on the C-terminal side of arginine residues. Family members have a preference for dibasic residues at the -1 and -2 positions while some also need a basic residue at the -4 position. These enzymes require divalent cations for activity, usually calcium, are inhibited by metal chelators but are not readily inhibited by traditional inhibitors of serine proteases. To confirm that the activity that cleaved PE was a furin-like enzyme, recombinant human furin was obtained from Genentech Inc. When PE, PEala281 and DT were mixed with human furin, the same degree of cleavage, the same pH optima, the same efficiency of fragment production and the same site of cleavage was noted with each substrate as had been seen with the protease from beef liver. Furin-cleaved PE also killed cells with greater rapidity than native PE. Also, an antibody to furin, raised against recombinant human furin reacted with both the protease from beef liver and the furin supplied by Genentech.

### Identification of a rate-limiting step in the toxicity pathway of PE.

PE is proteolytically cleaved within cells and is then reduced to generate a 37 kD C-terminal fragment which translocates to the cytosol and ADP-ribosylates elongation factor 2. Three recent studies have suggested that sequences at the N-terminus of the 37 kD fragment are important for toxicity. Two studies showed that tryptophan 281 is important for toxicity (Ogata et al. J Biol Chem 1992;267:25396-401, Kasturi et al. J Biol Chem 1992;267:23427-33). When residues 280-282 were changed individually to methionine, only the change at position 281 produced a toxin molecule with substantially reduced cytotoxic activity. When alanine scanning mutagenesis was performed on surface residues near the N-terminus of the 37 kD fragment, amino acids 280-2, 285-6 and 290 were individually changed to alanine and each mutant assayed for cytotoxic activity. Only the change of tryptophan 281 to alanine reduced toxicity by as much as 100-fold. And finally when a chimeric toxin composed of the 37 kD fragment fused with Transforming Growth Factor alpha (TGF $\alpha$ ) was tested for cytotoxic activity it had maximum activity when the protein started at residue 280 (Theuer et al. J Biol Chem 1992;267:16872-77). Activity was reduced 12-fold when amino acids 281-282 were deleted, 20-fold when amino acids 281-284 were absent and 200-fold when amino acids 281-288 were removed. Thus one or more specific residues at the N-terminus of the 37 kD fragment are important for toxicity.

In an effort to understand the requirement for particular amino acid side chains at position 281, additional mutations were generated at this residue. And because the side chains of amino acids 284 and 289 are clustered near the side chain of tryptophan 281, the importance of these amino acids was also investigated. Because all the mutations were located close to the Arg-Gly bond where PE is cleaved, mutant toxins were assayed for changes in susceptibility to proteolysis and in the patterns of fragments generated. Finally, a competition assay was devised to show that these three specific residues contribute to a saturable interaction with a cellular component.

We found that there was a 4- to 250-fold loss in toxic activity when tryptophan 281, leucine 284 or tyrosine 289 were changed to other residues. Mutations at these three positions did not interfere with the receptor binding, cell-mediated proteolytic cleavage or ADP-ribosylating activity. To determine the role of these amino acids, a competition assay was devised in which the addition of excess PE $\Delta$ 553, a mutant form of PE that lacks ADP-ribosylation activity, competed efficiently for the toxicity of PE. In this assay the addition of excess of PE with mutations at 281, 284 and 289 competed poorly. Further analysis revealed that competition by excess toxin occurred after proteolysis step since PEgly276, a mutant form of PE that is not cleaved appropriately by cells, did not compete. And competition did not depend on the presence of REDLK at the C-terminus of the toxin since PE with a deletion of the last 15 amino acids competed for PE toxicity as well as or better than PE $\Delta$ 553. We conclude that specific amino acids at the N-terminus of the 37 kD fragment interact in a saturable manner with an unknown intracellular component.

### Publications:

Ogata M, Fryling CM, Pastan I, FitzGerald DJ. Cell-mediated cleavage of *Pseudomonas* exotoxin between Arg279 and Gly280 generates the enzymatically active fragment which translocates to the cytosol, J Biol Chem 1992;267:25396-401.

Theuer CP, FitzGerald D, Pastan I. A recombinant form of *Pseudomonas* exotoxin directed at the epidermal growth factor receptor that is cytotoxic without requiring proteolytic processing, *J Biol Chem* 1992;267:16872-77.

Kasturi S, Kihara A, FitzGerald D, Pastan I. Alanine scanning mutagenesis identifies surface amino acids on domain II of *Pseudomonas* exotoxin required for cytotoxicity, proper folding and secretion into periplasm, *J Biol Chem* 1992;267:23427-33.

Kounnas MZ, Morris RE, Thompson MR, FitzGerald DJ, Strickland DK, Saelinger CB. The  $\alpha_2$ -macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes *Pseudomonas* exotoxin A, *J Biol Chem* 1992;267:12420-23.

Pastan I, Chaudhary VK, FitzGerald DJ. Recombinant toxins as novel therapeutic agents, *Annu Rev Biochem* 1992;61:331-54.

FitzGerald DJ, Pastan I. *Pseudomonas* exotoxin - recombinant conjugates as therapeutic agents, *Biochem Soc Trans* 1992;20:731-4.

FitzGerald D, Chaudhary VK, Kreitman RJ, Siegall CB, Pastan I. Generation of chimeric toxins. In: Franekl, AE, ed. *Genetically engineered toxins*. New York: Marcel Dekker, 1992;447-62.

Theuer CP, FitzGerald, DJ, Pastan I. Immunotoxins made with a recombinant form of *Pseudomonas* exotoxin A that do not require proteolysis for activity, *Cancer Res* 1993;53:340-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08758-02 LMB
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure and Mechanistic Study of <i>E. coli</i> RNA Polymerase and its Role in Clinical Applications		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)		
PI:	D. J. Jin	Senior Staff Fellow LMB, NCI
Others:	M. Xu	Special Volunteer 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 STAFF YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:
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.) The goals of this project are: (1) Determination of structural features of <i>E. coli</i> RNA polymerase (RNAP), particularly of its catalytic center and domains that interact with auxiliary factors, such as the various $\sigma$ factors. (2) Analysis of the biochemical mechanism of different steps of transcription by <i>E. coli</i> RNAP. (3) The screening and identification of antibiotics capable of inactivating rifampicin-resistant (Rif <sup>r</sup> ) RNAP for potential clinical use. The realization of this goal will depend upon the structural and mechanistic studies of <i>E. coli</i> RNAP.  Study of the nonproductive initiation has been focused. RNAP sometimes makes two kinds of nonproductive initiation products after the first phosphodiester bond formation and before entering into an elongation mode, which can be a rate-limiting step in transcription. One is abortive synthesis which is DNA-templated, the other is stuttering synthesis which is pseudo-templated. We have found that one Rif <sup>r</sup> RNAP mutant, RpoB3401, has reduced affinity for UTP and overproduced abortive initiation products at the <i>pyrBI</i> promoter. This result indicates the importance of Km of RNAP for nucleotide(s) in controlling the abortive initiation. We have found that wild-type RNAP also produces stuttering initiation products at the <i>galP2</i> promoter. The stuttering synthesis at <i>galP2</i> was sensitive to changes in UTP concentration and was repressed by transcription factor cAMP-CRP. A different Rif <sup>r</sup> RNAP mutant, RpoB3449, dramatically reduced its stuttering synthesis at <i>galP2</i> . These results indicate that the rif-region is important for both the abortive and stuttering synthesis in initiation.  A Rif <sup>r</sup> mutation from pathogenic bacteria <i>P. aeruginosa</i> was sequenced and found to have the same amino acid change as that in <i>E. coli</i> indicating that the rif-region is highly conserved in bacteria.  The target of several antibiotics including rifampicin is RNAP in bacteria. To test whether other antibiotics can inhibit Rif <sup>r</sup> RNAPs' function, we have determined the sensitivity of Rif <sup>r</sup> RNAPs to these antibiotics. Different degree of cross-resistance were found for different antibiotics. By this screening, we hope to identify and make suggestions in design of antibiotic for their ability to inhibit Rif <sup>r</sup> bacteria which has become a major problem clinically.		

Major Findings:

- I. Studying the biochemical mechanism of nonproductive initiation and the effects of Rif<sup>r</sup> RNAPs on this process.

This part of the work is to analyze the nonproductive synthesis in transcription initiation and to identify the sites in RNAP that are involved in this step.

A. Study of the effect of RpoB3401, a Rif<sup>r</sup> RNAP having a Arg529Cys change in the  $\beta$  subunit, on nonproductive initiation at the promoter of *pyrBI*, a pyrimidine biosynthetic operon. At the *pyrBI* promoter, wild-type RNAP regulates the efficiency of initiation by making more nonproductive stuttering initiation products at high concentrations of UTP than at low concentrations of UTP, an end product of the operon. Wild-type RNAP makes little abortive initiation products at this promoter. RpoB3401 behaved like wild-type in stuttering synthesis at the *pyrBI* promoter. However, different from wild-type RNAP, RpoB3401 overproduced abortive initiation products because it appeared to have reduced affinity for UTP during the transition from initiation to elongation. Kinetically, RpoB3401 is slower in binding of an incoming UTP during initiation compared to wild-type RNAP, thus shifting the reaction away from entering elongation and favorably to releasing aborted products. This result indicates that the  $K_m$  of an initially transcribed complex for nucleotide(s) is important for abortive synthesis. Since the *in vitro* effect of RpoB3401 on abortive initiation could account for the observed *in vivo* effect of the rpoB3401 mutation on initiation from the *pyrBI* promoter, this is the first documentation on the biological significance of abortive synthesis in gene expression. Moreover, because RpoB3401 affects only abortive synthesis but not stuttering synthesis, and 5'-Br-UTP suppressed the stuttering synthesis but not the defect of RpoB3401 in abortive synthesis, the mechanisms underlying the two kinds of nonproductive initiation, abortive and stuttering synthesis, are distinct. A manuscript entitled "An *Escherichia coli* RNA polymerase defective in promoter clearance due to its reduced affinity for uridine triphosphate" by Ding Jun Jin and Charles L. Turnbough, Jr. (Department of Microbiology, University of Alabama, Birmingham) is in the reviewing process. New RNAP mutants that have altered initiation activity at the *pyrBI* promoter will be isolated and studied.

- B. RNAP makes stuttering synthesis at the *galP2* promoter of the *E. coli gal* operon.

1. We have discovered that wild-type RNAP produced stuttering initiation products at the *galP2* but not *galP1* promoter. The stuttered products are pppAUUUU<sub>n</sub> ( $n \geq 1$ ) and is pseudo-templated from *galP2*. The stuttering synthesis from *galP2* was sensitive to changes in UTP concentration and was enhanced at higher UTP concentrations.
2. The stuttering synthesis from the *galP2* promoter was inhibited by cAMP-CRP complex. This result not only indicates that the stuttering synthesis can be controlled by a transcription factor but also unveils a new role for cAMP-CRP in gene expression.
3. The mechanism by which cAMP-CRP represses *galP2* is an unsolved issue. One working hypothesis has been that the bound cAMP-CRP becomes a steric hindrance

for RNAP binding to *galP2*. To test this model, we analyzed the initiation products with different initiating nucleotides or dinucleotides that are specific for *galP2* promoter and found that in the presence of cAMP-CRP, wild type RNAP appeared to be able to make one or two phosphodiester bonds from the *galP2* promoter indicating that cAMP-CRP does not exclude RNAP binding to the *galP2* promoter. We propose that cAMP-CRP represses *galP2* initiation at a step after the first phosphodiester bond formation, probably at a translocation step, an action analogous to the mode of action of rifampicin. We plan to pursue this study.

4. Cells in which *galP2* was fully active but *galP1* was minimal (*cya<sup>-</sup>galR<sup>-</sup>carA<sup>-</sup>*) had a slower growth rate at higher UTP concentration than at limiting UTP concentration in a medium containing galactose as sole carbon source. This result suggests that expression of the *gal* operon *in vivo* might also be sensitive to changes in UTP concentration. The good correlation between the *in vitro* and the *in vivo* results indicates that the stuttering synthesis at *galP2* has biological significance.
- C. RpoB3449, a Rif<sup>r</sup> RNAP having a Ala532 deletion in the  $\beta$  subunit, has reduced stuttering synthesis from the *galP2* promoter. This result indicates that part of the rif-region is also important for stuttering synthesis. The effect of RpoB3449 on *galP2* will be studied in detail to understand how stuttering synthesis is controlled.

## II. Identification of rif-region in pathogenic bacteria and study of cross-resistance of Rif<sup>r</sup> RNAP to other antibiotics.

This part of our work is an attempt to aim at overcoming clinical problems of emerging Rif<sup>r</sup> mutants of infectious microorganisms.

A. Rifampicin-containing antibiotic regimens were found to be effective in treatment of patients infected with *P. aeruginosa* and became bacteremia and pneumonia. However, emerging Rif<sup>r</sup> mutations of *P. aeruginosa* could hamper the treatment. To understand the nature of the Rif<sup>r</sup> mutations of *P. aeruginosa*, we have sequenced a Rif<sup>r</sup> mutation of *P. aeruginosa* and found to have the same amino acid change as that in *E. coli* indicating that rif-region is highly conserved in bacteria. The RNAP purified from the Rif<sup>r</sup> *P. aeruginosa* cells was resistant to rifampicin *in vitro* confirming that the identified mutation is responsible for the drug resistance.

B. Several antibiotics inhibit RNAP functions in bacteria. One way to probe whether the rif-region is overlapping with other antibiotic-binding sites in RNAP is to determine the sensitivity of each Rif<sup>r</sup> RNAP (total 12 different ones were purified) to these antibiotics. Two rifampicin derivatives, rifabutin and rifapentane have found to be complete cross-resistance with rifampicin. Both of the rifampicin derivatives have modified at 3'-position with different side group, indicating that the 3'-position of the rifampicin molecule is not involved in binding to RNAP. We are planning to screen for other rifampicin derivatives that have modifications at other positions of the rifampicin molecule. Half of the purified Rif<sup>r</sup> RNAPs were resistant to a different degree to a new antibiotic, sorangicin A which has a different structure than rifampicin. The partial cross-resistance suggests that the binding sites for rifampicin and sorangicin partially overlap. About half of the Rif<sup>r</sup> RNAPs showed slightly altered sensitivity to streptolydigin and one Rif<sup>r</sup> RNAP became partially resistant to

streptolydigin, indicating that partial sharing of the binding sites for these two antibiotics. Indeed, Rif<sup>r</sup> and Stl<sup>r</sup> mutations map close to each other but do not overlap in *E. coli*. Lipiarmycin inhibits all purified Rif<sup>r</sup> RNAPs, indicating that lipiarmycin binding site and rifampicin binding sites are totally independent. By this approach, we will identify and make suggestions in design of antibiotics for their ability to inhibit Rif<sup>r</sup> RNAPs. Since Rif<sup>r</sup> mutations are highly conserved in bacteria, the results from the study of *E. coli* Rif<sup>r</sup> RNAPs will have general application for other clinically important microorganisms.

Publications:

None.



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b>  Z01 CB 08759-02 LMB
<b>PERIOD COVERED</b> October 1, 1992 to September 30, 1993		
<b>TITLE OF PROJECT</b> ( <i>#0 characters or less. Title must fit on one line between the borders.</i> ) Molecular Modeling		
<b>PRINCIPAL INVESTIGATOR</b> ( <i>List other professional personnel below the Principal Investigator.</i> ) (Name, title, laboratory, and Institute)		
PI: Others:	B. K. Lee N. Kurochkina S.-H. Jung B. Madan Y. Sergeev J. Cammisa	Chief, Molecular Modeling Section Visiting Fellow Visiting Fellow Visiting Fellow Exchange Scientist Computer Specialist  LMB, NCI LMB, NCI LMB, NCI LMB, NCI LMB, NCI LMB, NCI
<b>COOPERATING UNITS</b> ( <i>If any</i> )  Molecular Biology Section, LMB, DCBDC, NCI		
<b>LAB/BRANCH</b> Laboratory of Molecular Biology		
<b>SECTION</b> Molecular Modeling Section		
<b>INSTITUTE AND LOCATION</b> NCI, NIH, Bethesda, MD 20892		
<b>TOTAL STAFF YEARS:</b>  6.0	<b>PROFESSIONAL:</b>  5.0	<b>OTHER:</b>  1.0
<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 <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK</b> ( <i>Use standard un-reduced type. Do not exceed the space provided.</i> ) <p>The main research interest of the Section is to use and develop theoretical means to study the forces that govern the structure and interaction of globular protein molecules, to predict the three-dimensional structure of these molecules, and to engineer protein molecules with improved properties. Following were accomplished: (1) The laboratory's general-purpose, graphics-oriented programs, GEMM, GPLOT, and SPLOT, have been updated and improved. (2) Proved, by means of simple computer simulations of pure liquids, that the hydrophobicity does not arise from the hydrogen bonding property of water. (3) Devised a theoretical scheme by which the change in stability of a protein molecule upon point mutation can be understood and estimated from the small molecule data on hydrophobicities. (4) A method was devised that identifies possible folding initiation sites of a protein molecule when only its sequence information is given and a method is being developed to predict the three-dimensional structure of these initiation sites. (5) Structural pattern recognition analysis was made on the <math>\alpha/\beta</math> barrel motif of the protein structure. All known protein structures that have this motif can be automatically aligned using these recognized patterns. These patterns will be used in the future to spot the <math>\alpha/\beta</math> barrel structural motif from the sequence of unknown proteins. (6) A pair of potential interchain disulfide bonding sites in the Fv fragment of the immunoglobulins was identified. These bonds are expected to stabilize the Fv fragment. These sites can be located for any immunoglobulin from the sequence alignment alone.</p>		

Major Findings:

## A. Software maintenance (with Mr. J. Cammisa)

The laboratory has three major home-grown, graphics-oriented programs that are useful for all modeling works. These are the molecular graphics program, GEMM, the manual sequence alignment program, SPLOT, and the mathematical graph plotting program, GPLOT. During the past year, we have identified and corrected a number of bugs in these programs and incorporated numerous little improvements. These incremental changes are time-consuming but a necessary part of the ordinary software maintenance procedure. A new Monte Carlo simulation program, BOSS, has also been purchased and installed.

## B. Hydrophobic effect and protein stability (with Dr. Bhupinder Madan)

The hydrophobic effect is generally considered to be one of the most important forces that govern the structure and interaction of all biological molecules. Unlike other forces, however, there is as yet no consensus on the physical origin of this effect. This makes it impossible to assess on a truly physical basis the stability of a protein molecule and the binding constant for the association of any two molecules. The goal of this project is to obtain a general understanding of the physical basis from which this effect arises, to obtain its magnitude and temperature dependence, and to assess its contribution to the stability of a protein molecule and to the binding constant of any two molecules.

The hydrophobic effect is most conveniently defined as the difference,  $\Delta G$ , of the free energy change upon transferring a small non-polar molecule from the gas phase to water, on the one hand, and to a non-aqueous solvent, on the other. The large magnitude of this difference has traditionally been considered to be due to the formation, in the aqueous solution, of an "iceberg-like" structure around the solute. We showed, by means of a couple of particularly simple simulations, that such a large  $\Delta G$  value can be obtained from a liquid which cannot form a hydrogen bond, thus proving that the excluded volume effect is sufficient to explain the hydrophobicity. A manuscript reporting this finding is now in preparation.

The hydrophobic contribution to the stability of a protein molecule can be measured by observing the change in stability when an internal hydrophobic side chain is replaced with another of a shorter length. When such measurements were made, however, it was found (1) that the value varied within a wide range and (2) that the values were generally much greater than those expected from the small molecule transfer data. In the past year, a thermodynamic scheme was devised, by means of which these mutation data can be understood in a coherent way. It was found by this analysis that the magnitude of the hydrophobic effect on protein stability is indeed given by the value obtained from the transfer data on small molecules, but that some sites in the protein are rigid, in which case a significant amount of additional destabilization occurs upon the mutation. This study constitutes the first direct demonstration of the importance and the magnitude of the hydrophobic effect on protein stability. This work has been published (#4 in the Publications list).

## C. Protein folding (with Dr. N. Kurochkina)

Compared to the number of proteins whose sequence is known, the number of proteins for which the structure is known is appallingly small. The need for a technique for obtaining the three-dimensional structure of a protein molecule from its amino acid sequence has perhaps never been

greater. The goal of this project is to develop an effective technique for predicting the three-dimensional structure of a globular protein molecule from its amino sequence alone. This is a difficult problem that requires a long term commitment with no prospect of frequent publications.

In the past year, we refined the method of using effective conformational entropy to the prediction of the early folding initiation sites. When tested against appropriate experimental data, obtained through the use of nmr and hydrogen-exchange combination, about 75% correlation could be found between the calculated entropy values and the degree of protection against hydrogen exchange in the A-state of the molecule. This work has been published (#2 in the Publications list). We are now attempting to predict the structure of these initiation sites by (1) generating a large number of three-dimensional structures at these initiation sites, (2) doing a cluster analysis to classify these structures into discrete categories, and (3) to compare the most popular cluster (one with most members in the group) with the actual structure in the folded state. Preliminary indication is promising.

This cluster analysis program was also used in order to remove the one manual step in the machine prediction of the structure of crambin. Here, we find that the cluster analysis was not sufficient. We are at present searching for some new principle, which will augment the cluster analysis procedure and successfully remove the manual intervention step.

#### D. Structural pattern recognition for $\alpha/\beta$ barrel motif (with Dr. Y. Sergeev)

The  $\alpha/\beta$  barrel is probably the most popular structural motif found in all known protein structures. The two dozen or so proteins that have this common structural motif have different functions and essentially no sequence homology among them. Although the  $\alpha/\beta$  barrel nature of their structure can easily be recognized, there are enough differences among the individual structures within the same structural motif that three-dimensional structural alignment is also difficult. Because of these properties, the  $\alpha/\beta$  barrel is a challenging case for automatic structural pattern recognition and structure-based sequence alignment procedures.

In the past year, we devised a new scheme, specific to the  $\alpha/\beta$  barrels, by which the structural pattern can be characterized and unambiguously aligned. A manuscript describing this work is currently being prepared. We expect that the common structural alignment that this procedure allows will help identify important structural characteristics that are common to all structures in this family. Since this characteristic should be encoded in the sequence, there is some hope that it can be recognized from the sequence information alone, in which case, we be able to recognize and predict the three-dimensional structure of this family of proteins. We will shortly embark on a program along this approach.

#### E. Modeling with immunotoxin (with Dr. S.-H. Jung)

The Fv fragment is the smallest unit of an immunoglobulin that still has the antigen binding function. The small size is important in many application that use antibodies, such as medical imaging and as a part of immunotoxins directed against solid tumors, because the small size makes it easier for the molecule to penetrate the relevant tissue.

The Fv fragment is a non-covalently associated heterodimer of the heavy and light chain variable domains. The molecule is unstable without some modification. A common practice is to connect

the two chains by means of a short peptide linker. Such single chain Fvs (scFv) are often fully active. However, the carboxy end of the linker is close to the CDR region of the molecule and may perturb the antigen binding with some antibodies. The two chains can also open up, while still connected to one another, in which case it is easy for each chain to unfold individually.

In order to circumvent these shortcomings with the scFv, we sought to find a site where an interchain disulfide bond can be introduced. We have found a pair of such sites in the framework region of the Fv, by using the molecular graphics program GEMM and other modeling tools. An immunotoxin using an antibody that contains one of these disulfide bonds has recently been synthesized in the Molecular Biology Section of the Molecular Biology Laboratory. The molecule is found to be as active as, and considerably more stable than, its single-chain counterpart. The manuscripts describing this work are in preparation.

#### Publications:

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Juretic D, Lee B, Trinajstic N, Williams RW. Conformational preference functions for predicting helices in membrane proteins, Biopolymers 1993;33:255-73.

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Brinkmann U, Lee B, Pastan I. Cytotoxicity of recombinant immunotoxins containing the V<sub>H</sub> or V<sub>L</sub> domain of monoclonal antibody B3 fused to *Pseudomonas* exotoxin, J Immunol 1993;150: 2774-82.

## SUMMARY REPORT

### LABORATORY OF CELL BIOLOGY

DCBDC, NCI

October 1, 1992 to September 30, 1993

The Laboratory of Cell Biology consists of the Molecular Cell Genetics Section (Michael M. Gottesman, Chief) and the Chemistry Section (Ettore Appella, Chief). The Chief of the Laboratory of Cell Biology supervises research emphasizing the molecular basis of drug resistance in cancer cells, the molecular basis of p53 suppression of malignant transformation, the biological role and mechanism of ATP-dependent and acid proteases, the process of melanogenesis, the mechanism of antigen processing, and studies on the regulation of translation of HIV RNA. Approximately 32 personnel working on 8 specific research projects have contributed to the progress outlined in this summary.

#### Resistance of Cancer Cells to Anti-Cancer Drugs

Human cancer cells selected for resistance to chemotherapy frequently show cross-resistance to many different anti-cancer drugs. Work in the Molecular Cell Genetics Section of the Laboratory of Cell Biology in collaboration with the Laboratory of Molecular Biology (Ira Pastan, Chief) has emphasized studies on resistance to natural product anti-cancer drugs (e.g., anthracyclines, vinca alkaloids, and etoposide) and *cis*-platinum. Cross-resistance to natural product drugs frequently results from expression of the *MDR1* gene which encodes the 170,000 dalton P-glycoprotein, an energy-dependent multidrug efflux pump. Major questions under study include the mechanism of action of this pleiotropic transporter and the development of ways to improve cancer treatment. Analysis of this multidrug transporter through photoaffinity labelling studies, mutational alterations, and kinetic studies of drug transport, has led to a model in which natural product hydrophobic drugs are removed directly from the plasma membrane. To test this model and to learn more about how energy is transduced to drive transport, P-glycoprotein has been purified and a drug-dependent ATPase activity can be demonstrated in reconstitution experiments. Retroviral vectors encoding the *MDR1* gene have been used to confer multidrug-resistance on bone marrow of mice resulting in selective advantage of transduced bone marrow cells *in vivo*. This approach could allow protection of human bone marrow during anti-cancer therapy and might be generally useful in gene therapy to give selective advantage to transduced cells.

#### Suppression of growth of malignant cells

Both cAMP and p53 are able to slow the growth of certain malignant cells. Work supervised by Michael Gottesman in the Molecular Cell Genetics Section has focused on the molecular basis for activation of cAMP-dependent protein kinase (PKA) by cAMP. Mutations in the regulatory subunit (RI) of cAMP-dependent protein kinase have been selected directly in tissue culture cells, or after mutagenesis of cloned RI subunits *in vitro*. Many mutations in RI are dominant negative mutants which allow growth of cultured cells in the presence of cAMP analogs or agents which raise cAMP levels, or in *E. coli* where co-expression of RI and the catalytic subunit of PKA inhibits growth in the presence of cAMP. These new dominant negative RI mutants are being used to create moveable genetic elements which ablate cAMP responses in recipient cells.

Under the supervision of Ettore Appella in the Chemistry Section the growth inhibitory effect of wild-type p53 is being studied. Expression of wild-type p53, which has a unique conformation and phosphorylation state, prevents cells from entering the S phase of the cell cycle and represses expression of *b-myb*, PCNA and DNA polymerase  $\alpha$  mRNAs. The phosphorylation sites of p53 have been analyzed and PK, a nuclear kinase which targets a number of key nuclear proteins, has been shown to

phosphorylate serines at position 15 and 37 *in vitro*, but only serine 15 *in vivo*. Mutants that change the codon for serine 15 to alanine are partially defective in ability to block cell cycle progression.

### Proteolysis

In the Molecular Cell Genetics Section, Michael Maurizi has been studying the substrate specificity of the ATP-dependent proteases Lon and Clp and the function of ATP hydrolysis in promoting protein degradation. The Clp protease has two subunits: Clp, which is the protease, and ClpA or ClpX, which hydrolyzes ATP. Synthetic peptides have been used to define two classes of peptide binding sites in *E. coli* Clp protease and to demonstrate substrate channeling and processive cleavage in the multisubunit complex. There are two ATP-binding domains in ClpA. ATP binding in domain I is required for assembly and stability of the complex of ClpA and ClpP, while domain II plays a role in promoting degradation of large proteins. In collaborative studies with Dr. Susan Gottesman, *in vivo* activity of ClpX to degrade the lambda O protein as well as several other specific proteins has been demonstrated. The Clp system in *E. coli*, therefore, appears to be a multicomponent system with a number of ATPase subunits and possibly proteolytic subunits that combine in unique ways to degrade specific substrates.

The specificity of Lon protease is being studied by purification of two physiological substrates, CcdA and RcsA. In collaboration with Michael Gottesman, a human mitochondrial homolog of Lon protease has been cloned which is 38% identical to the *E. coli* Lon protease.

Malignant cells secrete large amounts of cathepsin L, and smaller amounts of cathepsin B, a lysosomal acid protease with broad substrate specificity. To determine whether either of these proteases contributes to the metastatic potential of mouse B16 melanoma cells, the laboratories of Michael Gottesman and Vincent Hearing have evaluated their levels of expression, and no correlation has been found in a series of B16 melanomas of varying metastatic potential. The mechanism of secretion of cathepsin L has been studied in the Laboratory of Michael Gottesman by generating a series of deletions which indicate that an intact carboxy-terminus is needed for secretion.

### Melanoma and Melanogenesis

Melanin plays a critical role in protection against ionizing radiation, such as ultraviolet light, which not only induces a variety of skin cancers, but also promotes their growth. Vincent Hearing in the Molecular Cell Genetics Section has studied the regulatory controls of melanogenesis under normal basal conditions as well as responses induced in melanocytes by stimulation of differentiation; his laboratory has also examined the immune responses elicited by melanoma tumor growth and factors important to the abilities of those tumor cells to metastasize. A number of loci have now been cloned which encode proteins essential to melanin production and which interact to affect the quantity and quality of the melanin produced; interestingly, although those genes belong to the tyrosinase gene family and share determinants that allow their specific expression only in melanocytes, they respond uniquely to stimulation of differentiation. Those encoded proteins interact in a melanosomal complex to synergistically regulate melanogenesis, and modulate the structure and function of the melanin produced (e.g., its photoprotective and/or cytotoxic properties). Other studies have been directed at examining melanoma antigen and surface protease expression. One such antigen, termed B700, is a murine melanoma antigen that has a human analog also specifically expressed by human melanomas; a monoclonal antibody which specifically recognizes that antigen has proved effective as a unique diagnostic indicator of a wide variety of types of human melanomas and is under development as an immunodiagnostic tool.

### Antigen Processing

Research supervised by Ettore Appella in the Chemistry Section has utilized mass spectrometry for the structural characterization of peptides bound to Class I and Class II MHC molecules. The sequences of several peptides from a variety of Class I MHC molecules have been obtained. These peptides are largely 8-10 residues in length and bear anchor residues occupying the second residue

position from the N-terminus and at the C-terminal position of the peptide. In contrast, peptides presented by Class II MHC molecules are 16-18 residues in length and exhibit ragged N- and C-termini. Unlike Class I-derived peptides, crucial binding regions are contained in different portions of the peptide molecule and the groove on Class II molecules is open at both ends, consistent with structures described by Wiley and coworkers in which peptides are observed to project out of the antigen binding groove. A large percent of peptides bound to Class II MHC molecules are derived from self Class I molecules. The predominance of these peptides derived from MHC-related proteins may be relevant to the etiology of autoimmune diseases.

Additional studies in the Chemistry Section have highlighted the role played by a new transplantation antigen which has been isolated from a methylcholanthrene induced tumor, Meth A. Amino acid sequence analysis of N-terminal and internal peptides of the purified antigen clearly indicates that this protein is unrelated to the heat shock-related protein, gp 96, which has been previously reported to have tumor rejection activity. Anti-tumor specific T cell lines have been isolated and cloned from the peritoneal exudate cells of mice immunized to either Meth A or another antigenically unrelated Balb/c sarcoma.

Finally, the structure of the binding site of the p7 nucleocapsid protein of HIV has been elucidated. A stem-loop sequence in HIV-1 viral RNA plays a major role in the binding of p7, and this is important to repress 35S RNA translation and facilitate the formation of a core structure essential for infectivity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 03229-23 LCBGY
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 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)		
PI:	E. Appella	Medical Officer LCB, NCI
Other:	K. Sakaguchi D. Loftus	Visiting Associate IRTA Fellow LCB, NCI LCB, NCI
COOPERATING UNITS (if any) H. M. Grey and A. Sette, Cytel, San Diego, CA V. H. Englehard and D. F. Hunt, University of Virginia, Charlottesville, VA		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Chemistry		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0
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.) The role of mass spectrometry for the structural characterization of peptides bound to Class I and Class II MHC molecules has been established. Sequence analysis of peptides in mixtures prepared from immunoaffinity purified proteins has been achieved by the combination of microcapillary HPLC with electrospray ionization/tandem mass spectrometry. The sensitivity and resolving power of the method is particularly suited for the identification of peptides that are recognized by individual T-cells. The sequences of 15 peptides eluted from HLA-A2.1 and HLA-B7 have been obtained. A sequence alignment has implicated two to three peptide positions important for specific interactions with the Class I molecule, and their importance was confirmed using synthetic peptides. Computer modeling was used to understand the specificity of peptide binding and to predict how defined changes in peptide structure would affect it. This technique is capable of accurately predicting the characteristics of peptide interactions, and it may be valuable for predictions involving other HLA molecules. Peptides derived from the processing of normal cellular proteins have been implicated in the formation of epitopes recognized by alloreactive T-cells and we have recently identified such a peptide as the epitope for a murine HLA-A2.1 specific CTL. This same approach is expected to facilitate identification of peptides produced upon viral infection or cellular transformation.		
Peptides presented by Class II MHC molecules are 16-18 residues in length and exhibit ragged N- and C-termini. All of the peptides identified are derived from integral membrane proteins or secretory components. A large percent are derived from self Class I molecules and the Class II-associated invariant chain. The truncation at both the N- and C-termini indicated that the Class II molecules bind antigens from a pool of randomly generated peptides. Binding data on truncated peptides suggest that the peptide binding groove on Class II molecules is open at both ends. The predominance of peptides bound to Class II molecules that are derived from MHC-related proteins may be relevant to the etiology of autoimmune diseases linked to various alleles of Class II molecules. An investigation of the T-cell responses in autoimmune diseases should clarify the existing relationship between MHC restriction and disease pathogenesis.		



Major Findings:

## I. T-cell antigen recognition and major histocompatibility complex (MHC) antigens.

1) Cytotoxic T Lymphocytes (CTL) recognize host cells that express new antigens as a result of viral infection or transformation. CTL do not recognize such new antigens directly, but only in association with cell surface Class I molecules of the major histocompatibility complex (MHC). These new antigens are comprised of short peptides which are directly bound to the Class I MHC molecules. The 3-dimensional structures of different Class I MHC molecules have all shown a cleft on top of the surface in which these peptides bind. The peptide binding cleft is lined with many of the amino acid residues that vary among Class I alleles and control peptide binding specificity. A major goal of our studies has been to unravel the structural features that contribute to the peptide binding specificity of different Class I MHC molecules and to use this information to identify peptides that are recognized by individual antigen specific T-cells.

MHC Class I molecules bind to a complex mixture of small peptides that is difficult to separate and analyze by conventional biochemical methods. The use of microcapillary HPLC combined with electrospray ionization/tandem mass spectrometry has circumvented this problem. Direct sequence analysis of peptides in mixtures can be performed at the subpicomole level. We have applied this technique to the fractionation and sequence analysis of naturally processed peptides bound to human Class I MHC molecules, HLA-A2.1 and HLA-B7. Under the standard conditions used for the mass spectrometric analysis, we have detected approximately 2000 ions from both types of HLA molecules. The calculated masses range from a low of 737 to a high of 1423 and roughly 90% of the peptides are present at 15-150 femtomoles per  $10^8$  cells, corresponding to 90 - 900 different complexes per cell. To date, sequences have been determined for 15 peptides extracted from HLA-A2.1 and HLA-B7. The majority of these peptides are 9 residues long. The predominance of peptides of a single length allowed us to align the sequences and look for conservation in amino acids at each position. All but two of the 9-residue peptides associated with HLA-A2.1 contained Leu/Ile at position 2, but in the case of HLA-B7 the predominant residue at this position was found to be Pro.

Computer molecular modeling studies indicate that the differences in the structure of pocket B in the binding site of the Class I molecules are responsible for the different amino acid residues that predominate at position 2 of bound peptides. The residues at location 9 are all limited to those with aliphatic hydrocarbon side chains (Ala, Val, Leu and Ile). This position was modeled projecting downward into the F pocket. Examination of the rest of the sequences revealed that no other residues or chemical types were present at any position at frequencies greater than 50%, with the exception of position P3 in HLA-B7. This amino acid was found to be predominantly Arg. Modeling experiments suggest that Arg lies in the D pocket and both energetic analysis and direct binding experiments suggest that this interaction is a significant component of peptide binding.

In summary, our data indicate a common mode of interaction of peptides with two different human Class I molecules based on appropriate positioning of the amino and carboxyl termini, together with complementary interactions between P2 and the B pocket and P9 and the F pocket and, in the case of HLA-B7, between P3 and the D pocket.

2) The sequence of several naturally processed T-cell epitopes has been recently determined based only on the comparison of the elution times of synthetic peptides that correspond to the epitope with those of the naturally processed forms. However, the method requires knowledge of the protein from which the peptide is derived. It has been shown that it is possible to reconstitute cytolytic responses of Class I restricted T-cells *in vitro* using naturally processed peptides separated by HPLC and target cells of appropriate specificity. We have used micro-HPLC/mass spectrometry in conjunction with target cell sensitization to identify and sequence peptide epitopes recognized by HLA-A2.1 specific CTL. For our studies we have used a murine T-cell clone that recognizes an endogenous peptide in association with HLA-A2.1 expressed on human cells. Peptides were extracted from immunoaffinity

purified HLA-A2.1 molecules. After three successive chromatographies, the T-cell epitope was found in a single fraction. Mass spectrometric analysis of this fraction showed a peptide of 9 amino acids with no matches to a known sequence. A synthetic peptide corresponding to the determined sequence specifically reconstituted the epitope recognized by the T-cell clone. This finding reinforces the hypothesis that the strong T-cell response to allo-MHC molecules is dictated by the recognition of peptides presented by allogeneic MHC molecules. In addition, it demonstrates that mass spectrometry can be used to identify and sequence individual peptides within complex HPLC fractions. This type of analysis should facilitate the identification of epitopes that are recognized by alloreactive, tumor-specific and autoimmune T-cells. The relatively small number of cells required for analysis by mass spectrometry should also facilitate the accumulation of data from tissue samples as well as cells in culture, perhaps leading to the identification of tumor antigens and tissue-specific autoantigens.

3) MHC Class II molecules bind peptides derived from both exogenous proteins and from secretory or integral membrane proteins that are synthesized by the antigen presenting cell itself. Sequences for a large number of peptides eluted from Class II I-A<sup>b</sup>, I-A<sup>d</sup> and I-E<sup>b</sup> of the mouse and HLA-DR of the human have been reported; they consisted of 16 to 18 residues and had ragged amino and carboxyl termini. Binding experiments confirmed that all the isolated peptides had high affinity for the groove of the Class II molecule. Truncation experiments were carried out to determine if the longer Class II peptides were of optimal length for high affinity binding. Truncated analogs of the peptides that bind the I-A<sup>d</sup> Class II molecule were synthesized and tested for binding. The data obtained indicated that unlike Class I - derived peptides, crucial binding regions are contained in different portions of the peptide molecule, and that Class II naturally processed determinants are not of minimal size; they can be truncated as much as 7 residues without appreciable losses of their MHC binding capacity. In contrast to Class I bound peptides, which are predominantly nonamers, Class II peptides are larger and have a high degree of heterogeneity in length and in the N- and C-terminal ends. The finding that there are no peptides shorter than 13 or longer than 25 residues bound to Class II molecules suggests that these proteins would play an active role in antigen processing. Therefore, template-trimming to give unique termini is not necessary.

In order to determine the conformation of the peptides in the binding groove, an analysis of the tertiary structure of this complex is necessary. Two dimensional nuclear magnetic resonance analysis has been done on a complex of a sixteen amino acid cytochrome C peptide and the Class II I-E<sup>k</sup> molecule. Although  $\alpha$ -helical in the native cytochrome C protein and with no uniform structure in solution, the peptide is tightly bound to the I-E<sup>k</sup> molecule. For the first time, we have directly observed that residues Ala-88 through Arg-91 lie outside the peptide binding groove and that the side chains of residues Ile-95, Ala-96 and Ala-101 make contact with aromatic residues in the binding site. The side chain of Thr-102 appears to be relatively mobile as does that of Lys-99 which appears to contact the aromatic side chain of Tyr-97. Comparing these data with x-ray structures of several peptides bound to various Class I MHC molecules and a recent x-ray structure of a Class II - bound peptide, it is reasonable to postulate that Lys-103 of the cytochrome C peptide binds to Class II in a position equivalent to the F pocket of the Class I MHC binding groove. Thr-102 is directed toward the T-cell receptor in a similar manner to the penultimate residue in all the Class I MHC/peptide complexes. The side chains of Tyr-97 and Lys-99 are also exposed towards the open side of the MHC peptide binding groove, where contacts with the T-cell receptor can occur. From peptide - MHC binding studies it is thought that Gln-100 is important for peptide interactions with the Class II I-E<sup>k</sup> molecule, but we have no NMR evidence of the type of interaction that is established. Further structural analysis should generate more detailed information about the buried or exposed nature of all side chains of the bound peptide. Recently, x-ray crystallographic analysis of a peptide - Class II complex has clearly shown that the groove is open at both ends and some side chains occupy specific pockets which determine the binding specificity. Future structural analysis will establish the type of interactions which are essential for the T-cell receptor to recognize the MHC-peptide complex and initiate the signaling event for activation of the T-cell.

## II. Tumor Antigens

The immunogenicity of chemically induced sarcomas in tumor rejection assays has been attributed to their expression of antigenic determinants, commonly referred to as tumor-specific transplantation antigens (TSTA) or tumor rejection antigens (TRA). A major goal of tumor immunology is the identification of these antigens in the hope that this knowledge might facilitate the identification of human tumor antigens, and provide a more rational approach to tumor vaccines and immunotherapy.

An analysis of the tumor rejection inducing activity which can be isolated from the Con A Sepharose-binding protein fraction of the Meth A sarcoma indicated that this activity was associated with a Mr 110,000 glycoprotein, gp<sup>110</sup>. Together with evidence of restricted immunogenicity of Meth A gp<sup>110</sup> in tumor rejection assays, the results of the study strongly suggested that gp<sup>110</sup> might be the putative TSTA of chemically induced sarcomas.

An *in vitro* immunological-based assay system has been established to facilitate the further structural analysis of Meth A gp<sup>110</sup>. An anti-tumor specific CTL cell line was isolated and cloned from the peritoneal exudate cells (PEC) of Meth A -immune mice, and Meth A gp<sup>110</sup>, free of potentially bound proteins or peptides, was isolated from gp<sup>110</sup>-enriched fractions of Meth A sarcoma by high performance electrophoretic chromatography (HPEC). The proliferative responses of the anti-Meth A CTL cell line to HPEC-purified Meth A are consistent with the CTL cell line recognizing a gp<sup>110</sup>-related tumor-specific epitope. Future studies will focus on identifying and sequencing that portion of the gp<sup>110</sup> which encodes the CTL-recognized epitope. This information will then be utilized to establish the antigenic polymorphism of gp<sup>110</sup> expressed by chemically induced tumors and its role in inducing the highly restricted immunogenicity exhibited by these tumors, as well as to extend the analysis of gp<sup>110</sup> to human cancers.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 05597-04 LCBGY	
PERIOD COVERED October 1, 1992 to September 30, 1993			
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Biochemistry of Energy-Dependent (Intracellular) Protein Degradaton			
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)			
PI:	M. R. Maurizi	Research Microbiologist	LCB, NCI
Other:	M. W. Thompson	IRTA Fellow	LCB, NCI
	S. K. Singh	Visiting Fellow	LCB, NCI
	N. Wang	IRTA Fellow	LCB, NCI
COOPERATING UNITS (if any)			
LAB/BRANCH Laboratory of Cell Biology			
SECTION Molecular Cell Genetics			
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892			
TOTAL STAFF YEARS:	3.0	PROFESSIONAL:	3.0
		OTHER:	0.0
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.) ATP-dependent proteases play critical roles in the post-translational regulation of the amounts and activities of important cellular proteins and in the removal of damaged and denatured proteins from the cell. Our research has focussed on the biochemical characterization of the ATP-dependent Clp and Lon proteases from <i>E. coli</i> and on the discovery of new ATP-dependent proteases. Site-directed mutagenesis of the two ATPase sites in ClpA demonstrated that the active form of ClpA is a hexamer and that interactions between the subunits affect the ATPase and protease-activating activity of ClpA. The domain I site was shown to be involved in assembly of the ClpA hexamer, and interaction with ClpP and the domain II site was needed for activation of the proteolytic activity ClpP against large proteins. Model peptide substrates have been synthesized and shown to be cleaved more rapidly than any previous substrates. These peptides interacted tightly with ClpA and activated its ATPase activity. The site on ClpA occupied by the peptides defines the allosteric site for proteins on the enzyme, and occupancy of this site appears to be necessary to open the active site of ClpP for large polypeptides and to accelerate the catalytic cleavage of peptide bonds. Using partially inactivated ClpP, it was demonstrated that processive cleavage of polypeptides and proteins required the array of active sites present in the dodecameric form of ClpP. Processive cleavage of model peptides was observed when non-hydrolyzable analogs of ATP were used to activate the enzyme, indicating that ATP hydrolysis does not play a direct role in processivity. Studies done in collaboration with Dr. Susan Gottesman have demonstrated that another ATPase from <i>E. coli</i> , ClpX, is evolutionarily related to ClpA and that this protein functions <i>in vivo</i> with ClpP as an essential component of a proteolytic system that degrades the highly unstable lambda O protein. Sequence and biochemical data now indicate the presence in <i>E. coli</i> of four ATPases, Clps A, B, X and Y and suggest that the energy-dependent proteolytic systems of even this simple organism are highly complex. In studies done in collaboration with Drs. Michael Gottesman and Nan Wang, it has been found that a close homolog of the ATP-dependent Lon protease occurs in human mitochondria. Further biochemical and immunochemical studies are underway to identify the properties and function of this protease in human cells.			

Major Findings:

(1) Dr. Mark Thompson has synthesized peptide substrates for Clp protease. ClpP alone can cleave short hydrophobic peptides, but peptides longer than five amino acids were cleaved only when ClpP was activated by ClpA. Degradation of these longer peptides required ATP or a non-hydrolyzable analogs of ATP. We had previously shown that non-hydrolyzable analogs of ATP can also promote assembly of ClpA and interactions between ClpA and ClpP. Thus it appears that interaction between ClpA and ClpP is sufficient to open the active site of ClpP for longer peptides and to promote a more active conformation of ClpP. A series of peptides corresponding to portions of the propeptide removed during auto-catalytic maturation of ClpP *in vivo* were shown to be cleaved by ClpP and ClpA *in vitro* at the same methionine-alanine bond that is cleaved *in vivo*. Degradation studies with synthetic propeptides with altered amino acids indicate that the methionine at P1 position of the cleavage site can only be replaced by the hydrophobic amino acids, leucine and tryptophan. Hydrophobic residues in the P-2 to P-5 positions also appear to favor binding to ClpA and to accelerate cleavage of the peptide. Propeptide derivatives have high affinity for ClpA and activate the ATPase activity about 40-50%, similar to the activation seen with protein substrates. Binding of propeptide at the allosteric site is about about 100-fold tighter than at the protease active site, indicating that these two functional sites may be distinct and reflect different modes of interaction between protein substrates and the Clp protease. When the L-methionine was replaced by D-methionine at the cleavage site, the propeptide bound to the allosteric site of ClpA but was not cleaved. A longer polypeptide made from two propeptides in tandem was also cleaved by Clp protease at both available methionine-alanine bonds. Tandem propeptides with D-methionine at one of the potential cleavage sites were cleaved rapidly but only at the position containing the L-methionine.

ClpP subunits are arranged in two superimposed hexameric rings and thus there are potentially 12 active sites per protomeric unit. Chemical modification of the active site serines in ClpP with diisopropylfluoro phosphate (DFP) allowed preparation of ClpP with different numbers of active subunits per dodecamer. Such partially inactivated ClpP degraded small peptides and proteins but loss of protease activity required inactivation of more ClpP subunits than did loss of peptidase activity. Loss of protease activity was apparent only after 3-5 subunits were modified. Thus ClpP active sites appear to be heterogeneous and those sites used for peptidase activity appear to be more readily accessible to DFP. Untreated ClpP normally degrades proteins in a processive manner, cleaving substrates in multiple sites without release of large intermediates. Protein degradation by partially inactivated ClpP however resulted in partial cleavage of proteins and large polypeptides. With the tandem propeptide, significant amounts of intermediates with only one of the two available bonds cleaved were observed, in contrast to degradation by untreated ClpP in which both bonds are cleaved essentially simultaneously.

(2) Dr. S. K. Singh has constructed site directed mutations in the ATPase active sites of the two domains of ClpA and purified the mutant proteins to study their biochemical properties. Substitution of a glutamine for a critical lysine residue in domain I produced a mutant ClpA (K220Q) that could no longer assemble *in vitro* in response to ATP. This mutant had no activity in promoting the proteolytic activity of ClpP and had no ATPase activity, suggesting that assembly of the hexamer of ClpA is needed for expression of ATPase activity by domain II and for activation of ClpP. Substitution of an arginine for the lysine (K220R) produced an enzyme with reduced ATPase activity and reduced ability to activate ClpP. Addition of ClpA-K220Q to wild-type ClpA under reaction conditions caused inhibition of the ATPase activity of the wild-type enzyme, confirming that the active form of ClpA is an oligomer (probably a hexamer) and that interactions between subunits affect the activity of adjacent subunits. Mutations in the critical lysine of domain II (K501Q) produced ClpA that could assemble in the presence of ATP, confirming the essential role of domain I in the formation of active hexamers of ClpA. The K501Q mutant was able to associate into hexamers in the presence of ATP but had very low ATPase activity and was unable to activate ClpP proteolytic activity. The domain II mutant was able to

activate cleavage of the ClpP-propeptide, indicating that this mutant ClpA could interact with ClpP to open the active site for larger polypeptides but lacked the ability to promote cleavage of proteins. We conclude that the ATPase activity of domain II may be required for altering the conformation of protein substrates or for promoting movement of protein substrates bound to ClpA.

(3) In collaboration with Dr. Susan Gottesman, NCI, we have demonstrated the *in vivo* activity of another component of the Clp protease family and obtained additional evidence Clp-dependent degradation may involve multiple complexes containing different combinations of components. Genetic and biochemical studies have shown that ClpP degrades the highly unstable lambda O protein *in vivo*. This energy-dependent degradation activity is also dependent on another protein, called ClpX which is an ATPase related to but substantially different from ClpA. The ClpX gene has been cloned and sequenced (by W. P. Clark in Dr. Susan Gottesman's laboratory). We have prepared antibodies against purified ClpX and are using them to identify other related proteins in *E. coli*. *In vitro* activity of ClpP and ClpX on purified O protein was shown by Dr. Maciej Zylicz in Poland. Efforts are underway to obtain sufficient ClpX to analyze other degradative activities of ClpX and ClpP and to determine the interactions between these proteins and other Clp family members, particularly ClpA.

(4) In collaboration with Dr. Martine Couturier from Brussels and Dr. Susan Gottesman from NCI, we have initiated studies of the properties of the CcdA protein that determine its susceptibility to Lon-dependent degradation. CcdA is a low molecular weight F1-encoded protein that, by binding to the CcdA protein, prevents CcdA from inhibiting DNA gyrase and killing *E. coli* cells. We have purified CcdA by an improved method and found that the protein has a native tetrameric structure that is somewhat resistant to Lon protease *in vitro*. Earlier evidence for dissociated forms of CcdA resulted from proteolytic nicking during purification. Degradation of CcdA *in vivo* may depend on slight structural perturbations in CcdA or changes in its association with CcdB.

(5) In collaboration with Drs. Michael Gottesman, Susan Gottesman and Nan Wang, we have been investigating the activity and properties of a human homolog of *E. coli* Lon protease. A full length clone has been expressed in *E. coli* and the expressed protein has been partially purified. An ATP-dependent protein-degrading activity is associated with the partially purified protein. Antibodies raised against the 50 kDa carboxy terminal region of human Lon detect a 90 kDa protein in mitochondria isolated from cultured human neuroblastoma cells, and a similar protein is detected in whole cell extracts of other human cells. Immunofluorescence microscopy indicated that the major cross-reactive protein in human neuroblastoma cells was localized in mitochondria. Pulse labelling and immunoprecipitation indicate that the human Lon is made as a precursor of 95 kDa and rapidly processed *in vivo* to the mature 90 kDa form, presumably during uptake into mitochondria. The 100 kDa cloned human Lon protein was synthesized in a coupled *in vitro* transcription/translation system and was taken up into purified rat liver mitochondria in an energy-dependent process. Uptake *in vitro* was accompanied by processing to a size very similar to that of the mature Lon observed *in vivo*. Synthetic peptides corresponding to portions of the linear sequence of the human Lon have been synthesized. Antibodies against these peptides cross-react with the expressed human protein. Antibodies against a peptide with the highly conserved sequence surrounding the active site serine residue of Lon protease cross-reacts with both the expressed human protein and *E. coli* Lon protease and will be used to screen for homologous proteins in other organisms.

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Gottesman S, Maurizi MR. Regulation by proteolysis in prokaryotes and eukaryotes: energy-dependent proteases and their targets, *Microbiological Reviews* 1992;56:592-21.

Maurizi MR. ATP-dependent proteases. In: Bond, JS, ed. Proceedings of the 9th ICOP conference: Proteolysis and Protein Turnover. Williamsburg: 9th ICOP Conference: Proteolysis and Protein Turnover, 1993;in press.

Gottesman, S, Clark, WP, de Crecy V, Maurizi, MR. ClpX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*: sequence and *in vivo* activities, J Biol Chem 1993; in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 05598-04 LCBGY
PERIOD COVERED October 1, 1992 to September 30, 1993		
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. Gottesman	Chief, Laboratory of Cell Biology LCB, NCI
Co-PI:	I. Pastan	Chief, Laboratory of Molecular Biology LMB, NCI
COOPERATING UNITS (if any)		
	A. Nienhuis	Chief, Clinical Hematology Branch NHLBI, MH
LAB/BRANCH Laboratory of Cell Biology		
SECTION Molecular Cell Genetics		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
13.5	11.0	2.5
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		
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>We have continued to analyze the mechanism of action of the multidrug transporter and have worked on the development of new strategies to circumvent multidrug resistance in cancer and to exploit molecular knowledge of the multidrug transporter to design new cancer treatments. The multidrug transporter (P-glycoprotein) has been purified to near homogeneity and shown to be an active drug-dependent ATPase of high specific activity after reconstitution into proteoliposomes. Vesicles containing P-glycoprotein capable of transport have very active P-glycoprotein kinases, and this activity is stimulated by GTP. At least one novel plasma membrane associated P-glycoprotein-kinase has been partially purified, but its role in regulating activity of the multidrug transporter has not yet been determined. Kinetic studies demonstrate that the transporter interacts with drugs within the lipid bilayer, and indirect evidence suggests that drug may be removed from both the inner and outer leaflets of the bilayer. Molecular manipulation of P-glycoprotein by analysis of point mutations and chimeras with other members of the ATP-binding cassette (ABC) superfamily of transporters has revealed multiple regions of the molecule near or within the transmembrane domains which affect substrate specificity, and has indicated the interchangeability of ABCs between <i>MDR1</i> and <i>MDR2</i>, a related transporter of unknown specificity. Function of P-glycoprotein has been explored by insertional inactivation of the <i>mdr1b</i> gene in mouse adrenal Y-1 cells, with loss of ability of these cells to secrete steroids above basal levels. We have continued to develop the <i>MDR1</i> gene as a dominant selectable marker for gene therapy. Retroviral vectors expressing the human <i>MDR1</i> cDNA are able to confer resistance to taxol on transduced and transplanted mouse bone marrow cells, and this strategy is under consideration for gene therapy in humans to protect bone marrow during high dose chemotherapy for cancer. Two other multidrug resistant genetic systems are under development to aid in the analysis of other mechanisms of multidrug resistance: (1) A human melanoma line cross-resistant to epipodophyllotoxins (VP-16 and VM-26) and anthracyclines which has a deletion of Ala 428 in topoisomerase II; and (2) High level <i>cis</i>-platinum resistant human hepatoma and KB adenocarcinoma cells with multiple protein alterations.</p>		

Other Professional Personnel:

Y. Sugimoto	Special Volunteer	LCB, NCI
S. Zhang	Visiting Fellow	LCB, NCI
S. Ambudkar	Guest Researcher	LCB, NCI
B. Ni	Visiting Fellow	LCB, NCI
S. Goldenberg	Research Biologist	LCB, NCI
U. Germann	Visiting Associate	LCB, NCI
P. Schoenlein	Special Volunteer	LCB, NCI
D.-W. Shen	Visiting Associate	LCB, NCI
I. Aksentijevich	Senior Staff Fellow	LCB, NCI
F. Hsieh	Guest Researcher	LCB, NCI
C. Cardarelli	Research Biologist	LMB, NCI
J. Campain	Special Volunteer	LMB, NCI
G. Evans	Biotechnology Fellow	LMB, NCI
M. Siegsmond	Special Volunteer	LMB, NCI
J. Aran	Special Volunteer	LMB, NCI
T. Licht	Special Volunteer	LMB, NCI

Major Findings:

1. We have continued to purify P-glycoprotein from plasma membrane vesicles of human KB-V1 multidrug resistant cells and from insect Sf9 cells infected with an *MDR1* baculovirus. Both are rich sources of active P-glycoprotein capable of drug-dependent ATPase activity. The KB-V1-derived P-glycoprotein is approximately 95% pure after octyl glucoside extraction and fractionation on DEAE Sepharose CL6-B cellulose and wheat germ agglutinin columns. There is a low level of basal ATPase activity of these preparations in detergent. After reconstitution into liposomes prepared from *E. coli* lipids, phosphatidyl choline, phosphatidyl serine and cholesterol, this activity can be stimulated several-fold by drugs and is vanadate-inhibitible. Specific activities of pure P-glycoprotein of 15-25 umole of phosphate hydrolyzed per mg protein have been calculated. This specific activity is comparable to that of Na-K ATPase. This drug-stimulation appears to be specific for known substrates of P-glycoprotein such as verapamil and vinblastine, but other hydrophobic drugs which are not transported, such as camptothecin, do not stimulate the ATPase activity. The purified, reconstituted protein is photoaffinity labeled specifically by azidopine and forskolin. The purified material with a molecular weight of 160-170 kDa in detergent, copurifies with two P-glycoprotein fragments of 110 kDa and 55 kDa which represent the amino glycosylated half and the carboxy terminal half of P-glycoprotein which appear to be tightly non-covalently associated with each other during purification.

2. Because of published reports suggesting that P-glycoprotein may be regulated by phosphorylation, we have studied the phosphorylation of P-glycoprotein in transporting vesicles prepared from KB-V1 cells. These vesicles contain one or more protein kinases capable of phosphorylating P-glycoprotein in the presence of ATP or GTP. GTP dramatically stimulates phosphorylation by ATP in this crude vesicle system by an unknown mechanism. This phosphorylation is completely inhibited by staurosporine, but not by traditional inhibitors of protein kinase A or C, suggesting that it is due to a novel kinase or kinases. We have begun to purify one of the kinases from this vesicle system which is capable of phosphorylating purified P-glycoprotein preparations which are otherwise devoid of kinase activity. Preliminary results indicate that this partially purified membrane-associated kinase activity is not Ca-stimulated, but is inhibited by staurosporine, suggesting that it is a novel kinase which may play a significant role in physiological phosphorylation of P-glycoprotein.

3. In addition to these biochemical studies, we have continued a kinetic analysis of the uptake and efflux of drugs from multidrug resistant cells. These studies have been conducted on NIH 3T3 cells transfected with either a wild-type (G185) or mutant (V185) multidrug transporter which are expressed at approximately equal levels on the surface of these cells. Studies on the wild-type transporter indicate that hydrophobic drugs such as vinblastine, which are excellent substrates for the transporter, show decreased uptake from the earliest time points as well as increased efflux under conditions in which pump function is inhibited by energy deprivation to load the cells, and then re-energized. These studies support our model of P-glycoprotein as a "hydrophobic vacuum cleaner" capable of detection and ejection of drugs as they cross the plasma membrane, before they enter the cytoplasm. Interestingly, colchicine, a non-charged poorer substrate of P-glycoprotein, appears to be primarily effluxed by the pump, with little kinetic evidence of decreased uptake. The mutant transporter, in contrast, shows both decreased uptake and increased efflux of colchicine, but the initial rate of vinblastine uptake is higher than for the wild-type, consistent with the increased efficiency of transport of colchicine and decreased efficiency of vinblastine transport by the mutant. These results argue that there may be at least two components to detection of drugs in the membrane, consistent with removal from the inner or outer leaflets of the plasma membrane, or to multiple entry points along the transporter for drugs. In addition, inhibitors interact differently with mutant and wild-type transporters. For example, cyclosporine A very efficiently inhibits the mutant transporter compared to the wild-type, and verapamil is a more efficient inhibitor of the wild-type transporter.

4. Molecular genetic approaches have also been taken to study the mechanism of action of P-glycoprotein. Several chimeras and mutants have been shown to have altered function. A chimera formed when the first intracytoplasmic loop of *MDR2* (non-functional in drug transport) is substituted into the *MDR1* transporter results in loss of function, which can be returned by replacement of 4 out of 13 of the altered amino acids. This result argues that the *MDR2* transporter is not that different from *MDR1*, at least in this region. Chimeras in which different ATP-binding cassettes (ABCs) from *MDR2* and the cystic fibrosis transmembrane regulator (*CFTR*) have been substituted into *MDR1* suggest that *MDR2* has active ABCs, but that ABCs from *CFTR* are non-functional in *MDR1*. A deletion mutant lacking the amino acids encoded by exon 20 (residues 800-827) which removes part of the putative fourth intracytoplasmic loop is fully functional for transport of anthracycline and taxol, but has reduced transport of actinomycin D and Vinca alkaloids. These results argue that it should be possible to design multidrug transporters with specific alterations in drug specificity, and that some internal deletions in P-glycoprotein can be tolerated without major loss of function. They also suggest a certain compartmentalization of P-glycoprotein activities which affect substrate specificity, consistent with more than one site of drug-protein interaction.

5. To facilitate additional manipulations of the transporter for studies of mechanism and to design transporters with altered substrate and inhibitor specificity for gene therapy (see below), we have explored the use of model organisms for functional expression of P-glycoprotein. Studies in which human P-glycoprotein is expressed under control of the lambda P<sub>t</sub> promoter indicate that expression of the full-length *MDR1* cDNA is extremely toxic to *E. coli*, with induction of expression resulting in a rapid drop in viability and cessation of P-glycoprotein protein synthesis. Expression in *Saccharomyces cerevisiae* is, however, possible with at least two promoter-plasmid systems. P-glycoprotein in yeast membrane preparations is easily detected on Western blots, and binds azidopine in a specific manner. When P-glycoprotein is expressed in yeast strains sensitive to hydrophobic cytotoxic drugs, resistance to valinomycin, daunorubicin, and actinomycin D can be demonstrated. These studies suggest that yeast may be a suitable organism for molecular structure-function studies of P-glycoprotein.

6. Multidrug resistance encoded by the *MDR1* gene is a dominant selectable marker suitable for use *in vivo* as well as *in vitro*. Retroviral vectors using either a Harvey sarcoma virus promoter, or a Moloney promoter, have been constructed and a variety of producer cell lines (GP+E86, AM12) have been

selected which have high supernatant titers of transducing *MDR1* retrovirus ( $>1 \times 10^5$ /ml). These supernatants are free of non-defective virus and are therefore suitable for gene therapy experiments. Mouse bone marrow transduced with these retroviruses can be transplanted into host animals and continues to express the *MDR1* gene several months after transplant. After selection *in vivo* with taxol, *MDR1*-containing marrow cells become more prominent in the peripheral circulation, suggesting that the *MDR1* gene has conferred a selective advantage on the transduced marrow. The finding of long-term transplantable *MDR1*-expressing cells, and evidence for similar DNA insertion sites in peripheral cells of different lineages (lymphocytes and granulocytes), indicates that stem cell transduction has occurred in the mouse. Previous evidence from our *MDR1* transgenic mice, and from transduction of erythroleukemia cells suggests that relatively high level expression of the *MDR1* gene in bone marrow has little or no deleterious effect on function. These experiments, combined with the ease of selection of the multidrug resistant phenotype, cell surface expression of P-glycoprotein which allows for detection and sorting of living cells, and the potential ability to "design" unique transporters functionally distinguishable from the wild-type transporter, indicate that the *MDR1* gene is a good candidate for gene therapy in humans. Since protection of human bone marrow from the toxicity of anti-cancer therapy using the *MDR1* gene should be possible, clinical trials testing the feasibility of this approach in patients with breast and ovarian cancer undergoing autologous bone marrow transplantation are under consideration.

7. The ability of the *MDR1* gene to act as a selectable marker in gene therapy suggests that it could be used to select for the transfer of other genes into cells *in vivo*. We are currently designing vectors in which expression of non-selectable genes should occur after selection for multidrug resistance. Two approaches have been taken: (1) production of chimeric proteins, in which non-selectable genes are fused to the 3' end of the *MDR1* cDNA to produce bifunctional molecules. This approach has been previously shown to work with adenosine deaminase, which is fully functional when fused to P-glycoprotein; and (2) production of polycistronic mRNAs in which both P-glycoprotein and a second protein are separately encoded each with its own translation initiation region, separated by an internal ribosomal entry site derived from encephalomyocarditis virus. A variety of cDNAs including those encoding proteins defective in lysosomal storage diseases are being utilized for these studies.

8. To study the normal role of P-glycoprotein in transport of endogenous compounds, we have inactivated an *mdr* gene in cultured mouse adrenocortical Y1 cells. These cells synthesize and secrete steroids in large amounts, and they contain highest amounts of the *mdr1b* gene, with only small amounts of *mdr1a* and *mdr2* mRNA. We have prepared a homologous recombination vector with a G418 resistance cassette inserted into the *mdr1b* gene as a positive selection and one HSV-TK gene at each end of the *mdr1b* sequences to use for negative selection against non-homologous inserts. Approximately 1 of 150 G418 resistant Y1 clones had inserted the G418 resistance cassette into the center of the *mdr1b* gene, thereby inactivating one of two *mdr1b* alleles in this cell line. This insertion resulted in the inability of the mutant Y1 cells to secrete steroids above basal levels in response to ACTH. Paradoxically, the *mdr1b* mRNA from the second allele increased in amount approximately 10-fold, suggesting that this second allele encoded a functionally inactive transporter which was up-regulated by a feedback mechanism. These results suggest that P-glycoprotein may play an important role in secretion of steroids above basal levels.

9. We have selected human melanoma cells in the presence of VP-16 (etoposide) and a potent inhibitor of the multidrug transporter. We obtained mutants cross-resistant to the non-intercalative topoisomerase inhibitors VP-16 and VM-26, with lower levels of resistance to anthracyclines. As expected, resistant mutants do not express P-glycoprotein, but one series of mutant cell lines of increasing resistance express a mutant DNA topoisomerase II $\alpha$ . The mutation deletes amino acid 428 (an alanine). With increasing levels of resistance to VP-16, the relative percentage of mRNA encoding the mutant topoisomerase increases, although the total amount of mRNA and protein does not change, suggesting

that this mutation is responsible for the resistance to VP-16 and VM-26. Because this mutation is in a somewhat different region of topoisomerase II from other recently described mutants, and because nuclear extracts contain normal amounts and activity of topoisomerase II, we hypothesize that this mutation may affect an interaction of topoisomerase II with another component of the nucleus needed for sensitivity to anti-cancer drugs.

10. Mutants of human KB adenocarcinoma and human hepatoma cells resistant to high levels of *cis*-platinum have been isolated in multiple steps. Two dimensional gel electrophoresis of extracts of these cells show a large number of changes in proteins, but an increase in amount of a protein(s) of approximate molecular weight 52 kDa is consistently seen in both cell types. Expression of the 52 kDa protein(s) is stably increased in the absence of *cis*-platinum, and revertant cells which have lost *cis*-platinum resistance have reduced amounts of the protein(s). To characterize the genetic changes in the *cis*-platinum resistant hepatoma cells, we have transferred DNA from these cells to drug sensitive mouse fibroblasts and obtained *cis*-platinum resistant primary and secondary transfectants, with human Alu sequences linked to transfer of the resistance phenotype. Efforts to clone the genomic DNA associated with transfer of *cis*-platinum resistance are underway.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 05599-03 LCBGY
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Tumor Suppressor Protein, p53		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E. Appella	Medical Officer LCB, NCI
Other:	M. Fiscella	Visiting Fellow LCB, NCI
	K. Sakaguchi	Visiting Associate LCB, NCI
	N. Zambrano	Visiting Fellow LCB, NCI
	S. J. Ullrich	Special Volunteer LCB, NCI
COOPERATING UNITS (if any) W. E. Mercer, Jefferson Cancer Institute, Philadelphia, PA C. W. Anderson, Brookhaven National Laboratory, Upton, NY		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Chemistry		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0.0
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 <input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The product of the p53 gene suppresses cell growth and plays a critical role in suppressing development of human tumors. Wild-type (wt) p53 and its mutant form have been analyzed in an <i>in vivo</i> system in which the inducible expression of wt-p53 prevents cells from entering the S phase of the cell cycle. The growth arrest occurs near the restriction point in late G1 phase of the cell cycle and the expression of b-Myb, PCNA and DNA polymerase <math>\alpha</math> mRNAs was significantly repressed. Biochemical analyses indicated that the antiproliferative effect exerted by the wt-p53 correlates with the presence of a unique conformational state characterized by increased phosphorylation. To identify which p53 residues are phosphorylated, we examined DNA-PK's ability to phosphorylate synthetic peptides of the human p53 protein. Serines at positions 15 and 37 in the amino terminal domain of human p53 were phosphorylated. To prevent phosphorylation at these sites, mutants were constructed that changed the codons for Ser-15 or Ser-37 to alanine codons. Expression of p53 - Ala-37 blocked progression of the cells into S phase; however, p53-Ala-15 was partially defective in blocking cell cycle progression and caused a significant reduction in the steady-state level of p53.</p> <p>2-D phosphopeptide mapping showed that serines at positions 15, 9 and 33 in the amino terminal domain of human wt-p53 protein are phosphorylated <i>in vivo</i>. In two natural mutants from human tumors, phosphorylation at Ser-15 was reduced compared to the wt-p53. No changes were observed at the other sites. We suggest that phosphorylation of p53 at a specific site may contribute to the activity of wt-p53 to block cell growth.</p>		



### Major Findings:

The protein p53 was first discovered when expressed at high levels in chemically-transformed murine tumors and complexed with large T-antigen in SV40-transformed cells. Mutation or deletion of p53 is the most common genetic change associated with human malignancies. Most of the mutations identified thus far in human tumors are missense mutations located in the central region of the molecule resulting in the overexpression of mutant p53 protein. The overexpression of mutant p53 protein extends the *in vitro* growth potential of primary cells and converts established cell lines into highly tumorigenic lines. These characteristics suggest that p53 could be an oncogene. However, wild-type (wt) p53 cDNAs were found to be unable to cooperate with an activated ras oncogene in transformation and were found to be inhibitory to transformation induced by other combinations of nuclear oncogenes. This antiproliferative activity of wt-p53 appears to be due to its ability to arrest the growth of various transformed cells in the G1 phase of the cell cycle.

In order to more fully understand the mechanism of wt-p53 induced growth arrest, we have developed with Dr. Mercer a human cell line, GM47.23, which is stably transfected with wt-p53, and with expression that is under the control of the dexamethasone-inducible MMTV-promoter. Expression of wt-p53 induces G1 growth arrest in the GM47.23 glioblastoma cell line. The effect of overexpressing some of the mutant p53 proteins found in human tumors has also been investigated using the above inducible system. None of the mutant p53 proteins tested exhibited an antiproliferative effect. These results strongly suggest that naturally occurring missense mutations in the p53 gene are sufficient to inactivate the negative regulatory function encoded by the wt-p53 protein. Overexpression of wt-p53 protein in our model has been shown to down-regulate the expression of endogenous late G1/S phase genes encoding proliferating cell nuclear antigen (PCNA), b-myb and DNA polymerase alpha.

Evidence is rapidly accumulating to suggest the wt-p53 protein might function as a transcription factor, and it appears that wt-p53 acts in part as a DNA-binding protein. However, it is also likely that the wt-p53 protein stimulates transcription by protein-protein interaction between its activating domain and other components of the transcriptional apparatus. At present, it is not known whether down-modulation of the above endogenous cellular genes is a direct or indirect effect of wt-p53 and at what level down-modulation occurs. An attractive possibility is that wt-p53 may act directly to transcriptionally activate the expression of a set of cellular genes that encodes "repressor proteins" critically important for cell cycle control. Identification of these endogenous cellular genes that are targets of activation is essential to prove that wt-p53 protein is involved in transcriptional activation *in vivo*. A subtractive library is being analyzed between uninduced and induced GM47.23 cells to identify these genes. Recent studies have also indicated that overexpression of wt-p53 protein in our model up-regulates expression of the MDM-2 oncogene, which encodes a putative transcription factor.

The study of the wild type protein has been hindered by its low level of expression; however, the inducible expression of high levels of wt-p53 in the GM47.23 cells has allowed us to study its biochemical properties. We have shown that human wt-p53 exists in a unique conformational and phosphorylation state during growth arrest; wt-p53 was found to be more phosphorylated compared to mutant p53 and did not display the Pab/421 epitope. This form of wt-p53 was not complexed with the endogenous mutant p53. The endogenous mutant p53 protein is unable to assume the above changes and retains its reactivity with the Pab/421 antibody. These results argue for a mechanism in which the control of normal cell proliferation is dependent on the balance between different forms of the p53 protein.

Presently, considerable attention is being given to the study of the phosphorylation of key nuclear proteins. Regulation of the activities of these proteins occurs through phosphorylation and dephosphorylation mechanisms in some way connected with the timing mechanism of the cell cycle. We have devoted our attention to a nuclear kinase, DNA-PK, since it targets a number of key nuclear proteins including several transcription factors, the SV40 large tumor antigen and the p53 protein. We have examined DNA-PK's ability to phosphorylate synthetic peptides corresponding to human p53 sequences. Serines at positions 15 and 37 in the amino terminal transactivation domain were phosphorylated by DNA-PK. Phosphorylation of the conserved Ser-15 in human p53 peptides depended upon the presence of an adjacent glutamine and was inhibited by the presence of a nearby

lysine. Phosphorylation of recombinant wild-type mouse p53 was inhibited at high DNA concentrations, suggesting that DNA-PK may phosphorylate p53 only when both are bound to DNA at nearby sites.

Our data suggest that DNA-PK may have a role in regulating cell growth and indicates that phosphorylation of serine in DNA-bound p53 could alter p53 function. However, two critical questions need to be answered before any conclusion can be reached; 1) Does phosphorylation of Ser-15 and Ser-37 affect p53 expression or function? 2) Does DNA-PK phosphorylate these residues *in vivo* and is there any difference in the phosphorylation of wt and mutant p53?

We have constructed human p53 mutants with Ser-15 and/or Ser-37 changed to alanine. Mutants that express alanine at residue 15 in place of serine as the only change to the wt human p53 sequence caused a significant reduction in the steady-state level of p53 in human T98G cells transformed with mutant-expressing constructs. Changing Ser-37 to alanine had no discernable effect on p53 expression. p53-Ala-15 was partially defective in blocking cell cycle progression; however, p53-Ala-37 blocked cells from entry into S phase as efficiently as wt p53. The unexpected effects on p53 protein expression suggest that due to changing Ser-15 to alanine there is an additional mechanism through which phosphorylation may control p53 activity. In order to determine if the difference in phosphorylation of wt and mutant p53 found in GM43.23 cells was at specific sites within the protein, we have compared the phosphorylation sites *in vivo* using 2-D phosphopeptide mapping. Our results show that wt-p53 is phosphorylated to a greater extent at Ser-15 compared to most mutant p53 analyzed. No other specific site in mutant p53 is selectively decreased except for Ser-15. This finding contributes significant information on how p53 is regulated. A full understanding, however, may require identification of the other kinases that phosphorylate N-terminal residues, identification of the other components that interact with the N-terminus and, perhaps, development of new assays for biological function. These studies should provide useful and significant new information concerning the mechanisms involved in the regulation mediated by eukaryotic DNA binding proteins.

#### Publications:

Ullrich SJ, Mercer WE, Appella, E. Human wild-type p53 adopts a unique conformational and phosphorylation state *in vivo* during growth arrest of glioblastoma cells, *Oncogene* 1992;7:1635-43.

Ullrich SJ, Anderson CW, Mercer WE, Appella E. The p53 tumor suppressor protein - A modulator of cell proliferation, *J Biol Chem* 1992;267:15259-63.

Lin D, Shields MT, Ullrich SJ, Appella E, Mercer WE. Growth arrest induced by wild type p53 protein blocks cells prior to or near the restriction point in late G1-phase, *Proc Natl Acad Sci USA* 1992;89:9210-14.

Mercer WE, Ullrich SJ, Shields MT, Lin D, and Alder H. Cell cycle effects of microinjected antisense oligonucleotides to p34<sup>cdc2</sup> kinase, *Annals NY Acad Sci* 1992;600:209-18.

Lees-Miller SP, Sakaguchi K, Ullrich S, Appella E, Anderson CW. The human DNA-activated protein kinase phosphorylates serine 15 and 37 in the aminoterminal transactivation domain of human p53, *Mol Cell Biol* 1992;12:5041-49.

Duthu A, Debuire B, Romano J, Erhart JC, Fiscella M, E, Appella E, May, P. p53 mutations in Raji cells: characterization and localization relative to other Burkitt's lymphomas, *Oncogene* 1992;7:2161-67.

Fiscella M, Ullrich SJ, Zambrano N, Shields M, Lin D, Lees-Miller SP, Anderson CW, Mercer EW, Appella E. The mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression, *Oncogene* 1993; 8:1519-28.

Ullrich SJ, Sakaguchi K, Lees-Miller SP, Fiscella M, Mercer EW, Anderson CW, Appella E. Phosphorylation at Ser-15 and Ser-392 in mutant p53 molecules from human tumors is altered compared to wild-type p53, Proc Natl Acad Sci USA 1993;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 08705-17 LCBGY
PERIOD COVERED October 1, 1992 to September 30, 1993		
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. Gottesman	Chief, Laboratory of Cell Biology LCB, NCI
Other:	S. Goldenberg M. Gosse	Research Biologist LCB, NCI Senior Staff Fellow LCB, NCI
COOPERATING UNITS (if any)		
	G. Merlino C. Jhappan	Research Microbiologist LMB, NCI Senior Staff Fellow LMB, NCI
LAB/BRANCH Laboratory of Cell Biology		
SECTION Molecular Cell Genetics		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
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<input type="checkbox"/> (a2) Interviews		B
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Cyclic adenosine-5'-monophosphate (cAMP) plays an important role in cell differentiation and malignant transformation. We have previously shown by generating a series of mutants of Chinese hamster ovary (CHO) cells that virtually all of the effects of cAMP on CHO cells are mediated through cAMP-dependent protein kinase. Dominant negative mutants affecting the regulatory (RI) subunits of this kinase are easily obtained in this system, since holoenzyme cAMP-dependent protein kinase normally consists of two RI and two catalytic (C) subunits in which the activity of the C subunits is inhibited by RI. Any mutation which blocks the ability of RI to interact with cAMP, thereby inhibiting the ability of cAMP to dissociate the complex, which releases active C, or which increases the association of RI and C will act as a dominant negative mutant and block cAMP effects. A similar ablation of the effect of cAMP can be achieved with vectors which allow overexpression of yeast phosphodiesterase, which degrades cAMP as it is formed. To study the interaction of RI with C we have expressed the Chinese hamster recombinant RI and C proteins in <i>E. coli</i> and demonstrated that active, interactive subunits can be synthesized in this system. A variety of mutations which affect cAMP binding to RI and interaction of RI with C have been characterized in this system. In addition, we have discovered that co-expression of RI and C in <i>E. coli</i> results in growth inhibition of the bacteria. This growth inhibition requires functional RI and active C as well as cAMP, and hence mimicks cAMP effects in mammalian cells. We have used this system to begin to isolate mutants of RI with the goal of generating a detailed map of functional and non-functional regions of this important regulatory protein. Furthermore, we have developed an expression system for mutant RIs which allows direct selection of dominant negative RI mutants in mammalian cells. Finally, efforts to overexpress yeast phosphodiesterase in transgenic mice have led to an unexpected finding: one of our transgenic mice is severely immunodeficient when bred as a homozygote, suggesting insertional inactivation of a gene essential for normal development of the immune system.		

Major Findings:

1. Cloned Chinese hamster regulatory (RI) and catalytic (C $\alpha$ ) subunits of cAMP-dependent protein kinase were expressed as soluble, active, and interactive proteins after high level expression in the *E. coli* Studier expression system. Six mutant RI proteins were analyzed biochemically in this system. Two mutations, GE201 and YF371 were identical to mutations previously engineered in mouse and bovine proteins and had the expected effect of reducing cAMP binding to RI. A new point mutation, WR262, had a similar effect, as did a carboxy-terminal nonsense mutations which deleted both cAMP binding sites (GStop200). One mutation, VG376, had no discernible effect on RI function, while VA89, a mutation in the putative RI-C interaction site, reduced binding of RI and C. Although this system will be useful for biochemical analysis of mutants generated in other systems (see below), the random generation of RI mutants is not an efficient system for structure-function studies of cAMP-dependent protein kinase.
2. To improve the efficiency with which functional mutants of RI and C could be recovered, we explored the possibility that expression of holoenzyme cAMP dependent protein kinase would have a selectable phenotype in *E. coli*. Co-expression on a polycistronic message of these two subunits results in very slow growth of the bacteria (microcolony phenotype). This phenotype requires: (1) active RI, as shown by failure of a mutant RI with reduced cAMP binding to inhibit growth when co-expressed with C; (2) active C, as shown by failure of a C subunit with a mutant ATP site to support the phenotype; and (3) the presence of cAMP, as shown by a requirement for adenylate cyclase or added cAMP in the host bacterium. When RI is mutated *in vitro* by PCR-directed mutagenesis and introduced into the RI-C polycistronic expression vector, bacteria which grow well can be readily selected. A substantial percentage of these appear to carry intact plasmids which express RI and C. In one case, sequencing of the RI indicated the presence of two mutations in the cAMP binding sites consistent with the observed phenotype. This system holds promise for the generation of a library of RI mutants to analyze structure-function relationships in this important protein.
3. In a second approach to generate selectable dominant negative mutants of RI for potential use as moveable elements to inactivate cAMP effects in mammalian cells and transgenic animals, we have developed a CMV-based vector system (pRc/CMV) for selection of mutant RI's in CHO cells. The approach is to mutagenize RI with PCR under conditions where one deoxynucleotide is limiting. The mutagenized library of RIs is inserted into pRc/CMV followed by electroporation of CHO cells and selection for G418 resistance and resistance to cholera toxin, which raises cAMP levels and inhibits growth of CHO cells. CHO mutants carrying pRc/CMV which are resistant to cholera toxin were readily selected, and the RI cDNA from one such transfectant was rescued by PCR and several independent clones were sequenced. Four mutations were found, three of which are within previously defined cAMP binding sites. The relative contributions of these mutations to the cAMP-resistant phenotype are being analyzed. This approach promises to allow selection of RI subunits which are more powerful dominant negative mutants than those previously available, possibly by resulting in multiple mutations within the RI coding region.
4. We have attempted to create transgenic animals in which cAMP effects were ablated in specific tissues in order to determine the requirement of cAMP for normal development. One attempt involved expression of a yeast phosphodiesterase (PDE) gene under control of an insulin promoter. The yeast PDE gene had been previously shown to ablate cAMP responses in CHO cells by degradation of cAMP as it is generated by adenylate cyclase. No animals were obtained in which expression of PDE was readily detected; however, one line of transgenic mice showed profound immune system defects when bred as homozygotes. These animals lack mature B and T cells, and develop undifferentiated lymphomas at an early age. Characterization of this profound immune system defect, which we believe probably results from insertional mutagenesis in a gene essential for normal immune development since yeast PDE mRNA or activity cannot be demonstrated, is in progress.

Publications:

Chin K-V, Chauhan SS, Abraham I, Sampson K, Krolczyk A, Wong M, Schimmer B, Pastan I, Gottesman MM. Reduced mRNA levels for the multidrug-resistance genes in cAMP-dependent protein kinase mutant cell lines, *J Cell Physiol* 1992;152:87-94.

Gosse M, Padmanabhan A, Fleischmann RD, Gottesman MM. Expression of Chinese hamster cAMP-dependent protein kinase in *Escherichia coli* results in growth inhibition of bacterial cells, *Proc Natl Acad Sci USA* 1993;in press

Gosse M, Fleischmann R, Marshall Mallika, Wang N, Garges S, Gottesman MM. Bacterial expression of Chinese hamster regulatory type I and catalytic subunits of cyclic AMP-dependent protein kinase and mutational analysis of the type I regulatory subunit, *Biochem J* 1993;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 08715-15 LCBGY
PERIOD COVERED October 1, 1992 to September 30, 1993		
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. Gottesman	Chief, Laboratory of Cell Biology LCB, NCI
Others:	S. Goldenberg	Research Biologist LCB, NCI
	J. Reed	Medical Staff Fellow LCB, NCI
	V. Hearing	Research Biologist LCB, NCI
	K. Urabe	Visiting Fellow LCB, NCI
	W. Vieira	Microbiologist LCB, NCI
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Molecular Cell Genetics		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0
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		B
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Malignantly transformed cells secrete a variety of proteases and protease precursors which have been hypothesized to be involved in the invasiveness, metastasis, immune suppression, and paraneoplastic syndromes associated with cancer. Our work has concentrated on the function of a secreted lysosomal cysteine propeptase called procathepsin L or MEP (for major excreted protein of transformed cells). Cathepsin L is a broad spectrum acid protease found both in lysosomes and secreted as the proenzyme into the extracellular space. We have cloned the genes for mouse and human procathepsin L and compared their structures and modes of regulation. Each has 8 exons and 7 introns located at the same positions within the coding region, but the structural gene for procathepsin L in the mouse spans 7400 bp, while the human procathepsin L gene is 5100 bp, with the size difference accounted for almost entirely by intron size. Unlike the mouse gene which has a unique start site of transcription and a single major mRNA species, at least two major mRNAs are transcribed from the human procathepsin L gene. These two mRNAs differ in their 5' untranslated sequences, and represent alternative splicing or alternative promoters. To determine why procathepsin L is secreted in large amounts by the activity of malignantly transformed cells and other cells which overproduce it, we have initiated a deletion analysis of the human procathepsin L cDNA. Expression of deleted forms of human procathepsin L in mouse NIH 3T3 cells results in either failure to form a stable structure (deletions of the "pro" piece) or failure to be secreted (carboxy-terminal deletions). To evaluate the function of secreted procathepsins in metastasis we have compared the synthesis, secretion, and activity of cathepsins L and B in a series of mouse B16 melanoma cells of varying metastatic potential, and find no clear correlation with ability to metastasize.</p>		

Major Findings:

1. The human procathepsin L structural gene has been isolated by use of exonic PCR primers derived from the sequence of a human procathepsin L cDNA. The gene spans 5100 bp, and consists of 8 exons and 7 introns. By analysis of mouse human hybrid cell lines using PCR primers specific for human cathepsin L, and by *in situ* hybridization to metaphase human chromosomes, we have mapped the cathepsin L gene to chromosome 9q21-22. Interestingly, there is a homologous gene on chromosome 10, which accounts for earlier reports mapping cathepsin L to this chromosome. In addition, there is at least one procathepsin L pseudogene which contains stop codons within the procathepsin L coding sequence. All human tissues and tumors tested contain at least two mRNAs for cathepsin L which differ at their 5' non-coding ends, with differences apparent just upstream from the translation-initiating ATG. One of these mRNAs includes sequences from the first intron of procathepsin L, as defined for the second mRNA. These two mRNAs result either from activity of two promoters (one of which would be in the first intron) or variable splicing of mRNAs derived from a single upstream promoter. Although the structure of these two mRNAs suggests that their efficiency of translation should be different, to date no differences in the relative abundance of these two mRNAs in different tissues or cancers have been detected.
  
2. Procathepsin L (MEP) is secreted in large amounts by cells which overproduce it. The secreted proenzyme appears to have intact Man 6-phosphate residues which allow lysosomal targeting, and most other lysosomal proteins are not secreted by these transformed cells. Hence, we searched for residues within the primary sequence of procathepsin L which might account for the efficiency of its secretion. Deletions were constructed within the cDNA for human procathepsin L. These were engineered into an expression vector consisting of an SV40 promoter and multiple *lac* operators which allowed high level expression in mouse NIH 3T3 cells expressing a chimeric *lac* repressor-VP-16 activator (the "LAP" system). Since antibody to human cathepsin L does not cross-react significantly with mouse cathepsin L, expression of the human cathepsin in the mouse cells was easily visualized and subcellular localization of this protein could be determined. Deletions within the "pro" region of procathepsin L resulted in either no detectable protein, or protein retained within the endoplasmic reticulum, suggesting that these sequences are needed for proper folding of the molecule. Deletions at the carboxy-terminus of procathepsin L had a profound effect on secretion, but did not affect targeting to lysosomes. These results suggested either that such sequences were essential for directing procathepsin L to the secretory pathway, or that the presence of such sequences interfered with normal lysosomal localization, with secretion being a default pathway for procathepsin L which did not go to lysosomes. To distinguish these two possibilities, we treated cells with tunicamycin, which blocks addition of high mannose carbohydrates and thereby prevents addition of the Man 6-phosphate lysosomal targeting residues. Full-length procathepsin L was mostly secreted under these conditions as expected, but the carboxy-terminal deletions remained in the cell, with at least some procathepsin L located in lysosomes. These results define three potential pathways for newly synthesized procathepsin L: (1) Man 6-phosphate dependent lysosomal localization; (2) Man 6-phosphate independent lysosomal localization by an as yet undefined pathway; and (3) Secretion dependent on signals embedded in the carboxy-terminus of procathepsin L.
  
3. Because of the high specific protease activity of secreted procathepsins against proteins which make up the basement membrane and extracellular matrix, we have begun to explore the role of procathepsin L and B in invasiveness and metastasis. In collaboration with the laboratory of Dr. Vincent Hearing, we have determined the levels of mRNA, and proteolytic activity of cathepsin B, levels of mRNA and protein synthesized and secreted for cathepsin L, and activity of urokinase in a series of mouse B16 melanoma cells of varying metastatic potential. These cells are unselected subclones whose ability to form melanotic nodules in mouse lungs after tail vein injection varies by approximately two orders of magnitude. In our initial studies, there is no obvious correlation between levels of these proteases and metastatic potential, but these studies are difficult to interpret since activity of secreted procathepsins and



urokinase in metastasis would be expected to depend not only on total amounts of mRNA and protein, but on cell surface and extracellular activity which reflects localization and the activity of endogenous and exogenous inhibitors for these proteases which are ubiquitous. To obtain more definitive conclusions about the role of these proteases in metastasis, we are attempting to "knock-out" these genes in mouse B16 melanoma cells, which appear to be diploid for both cathepsin L and B. Knock-out vectors for both cathepsin L and B have been constructed in a new homologous recombination vector system which we developed based on the positive (G418)-negative (HSV-TK, ganciclovir) selection system originally suggested by Capecchi.

Publications:

Chauhan SS, Popescu NC, Ray D, Fleischmann R, Gottesman MM, Troen BR. Cloning, genomic organization and chromosomal localization of human cathepsin L, *J Biol Chem* 1993;268:1039-145.

Chauhan SS, Gottesman MM. Construction of a new universal vector insertional mutagenesis by homologous recombination, *Gene* 1992;120:281-6.

Gottesman MM. Cathepsin L and cancer. In: Bond JS, ed. Proceedings of the 9th ICOP conference: Proteolysis and Protein Turnover. Williamsburg: 9th ICOP Conference: Proteolysis and Protein Turnover, 1993;in press.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b>  Z01 CB 09100-08 LCBGY
<b>PERIOD COVERED</b> October 1, 1992 to September 30, 1993		
<b>TITLE OF PROJECT</b> (80 characters or less. Title must fit on one line between the borders.) Immunogenicity of Melanoma		
<b>PRINCIPAL INVESTIGATOR</b> (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: V. Hearing	Research Biologist	LCB/NCI
Other: K. Urabe	Visiting Fellow	LCB/NCI
T. Kobayashi	Guest Researcher	LCB/NCI
P. Aroca	Guest Researcher	LCB/NCI
D. Gersten	Guest Researcher	LCB/NCI
L. Law	Scientist Emeritus	LG/NCI
J. Muller	Research Biologist	CBER/FDA
<b>COOPERATING UNITS</b> (if any) See the following page.		
<b>LAB/BRANCH</b> Laboratory of Cell Biology		
<b>SECTION</b> Molecular Cell Genetics		
<b>INSTITUTE AND LOCATION</b> NCI, NIH, Bethesda, Maryland 20892		
<b>TOTAL STAFF YEARS:</b> 3.8	<b>PROFESSIONAL:</b> 2.8	<b>OTHER:</b> 1.0
<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 <input type="checkbox"/> (a2) Interviews		
<b>B</b>		
<b>SUMMARY OF WORK</b> (Use standard unreduced type. Do not exceed the space provided.) <p>           This project is aimed at characterizing parameters important to the growth and differentiation of melanocytes and their significance to such critical properties of transformed melanocytes (termed malignant melanoma) as their ability to grow as primary tumors and to metastasize. Our studies have identified, isolated and characterized several distinct melanogenic enzymes that interact to regulate the quality and quantity of pigment produced within melanocytes. These proteins are encoded within a family of tyrosinase-related genes that are specifically expressed by mammalian melanocytes. Interestingly, although expression of these genes is specific to pigment producing tissues, they are independently regulated following stimulation of differentiation. We have shown that the phenotypic properties of the melanins produced by these catalytic regulatory controls can differ dramatically and the effects on the functional and photoprotective properties of these melanins are being actively studied. Our laboratory has also continued its interactive collaborations on the characterization of melanoma-specific antigens that are abnormally expressed on transformed melanocytes and which play a role in the host's immune responses to tumor growth. Monoclonal antibodies specific for one of those antigens, termed B700, have now been shown to be cross-reactive with human melanoma and have proven to be a highly specific probe for detecting malignant melanoma. The B700 antigen has been shown to be the major active antigen in a melanoma vaccine being developed that has proven to be efficacious in a model for spontaneous metastasis of murine melanoma.         </p>		

Outside Collaborating Units:

Elieser Gorelik, Pittsburgh Cancer Institute, Pittsburgh, PA  
 Koichiro Kameyama, Kitasato Institute Medical Center, Saitama, Japan  
 Richard King, University of Minnesota, Minneapolis, MN  
 Giuseppe Prota, University of Naples, Naples, Italy  
 David Shrayar, Brown University, Providence, RI  
 Francisco Solano, University of Murcia, Murcia, Spain  
 Richard Spritz, University of Wisconsin, Madison, WI  
 Daniel Vlock, Brigham and Women's Hospital, Boston, MA  
 Alison Winder, Glaxo Group Research Limited, Middlesex, United Kingdom

Major Findings:

1. Melanogenesis. Our studies have continued into the regulatory mechanisms involved in the basal differentiation of mammalian melanocytes and in their ability to respond to stimuli of differentiation, such as ultraviolet light or melanocyte stimulating hormone (MSH). We have now generated specific antibodies against synthetic peptides which recognize four distinct melanogenic proteins; those antibodies have been used to purify the corresponding proteins by immune-affinity chromatography and subsequently characterize their novel catalytic functions in melanogenesis. In combination with specific nucleotide probes for those genes, we have been able to characterize the sequence of events involved in the response mechanism of melanogenic stimulation at the transcriptional, translational and post-translational levels. We have found that although those four genes are closely related (belonging to a tyrosinase gene family) and are specifically expressed only by melanocytes, their transcriptional levels are differentially stimulated following treatment with MSH. The genes involved are encoded at the following murine loci: *albino* encodes tyrosinase, a trifunctional enzyme absolutely required for melanogenesis; *brown* encodes TRP1, which has a specific function as DHICA oxidase; *slaty* encodes TRP2, which has a specific function as DOPAchrome tautomerase; *silver* encodes Pmel17, which appears to function as an inhibitor of melanogen oxidation and/or polymerization. All four of these genes have human homologues which have also now been cloned and shown to be similarly specifically expressed by human melanocytes, and mutations at two of those loci (*albino* and *brown*) elicit various types of human albinism. We (with Spritz) have now reported the specific lesions elicited by many of those human albino mutations and characterized the catalytic function that is disrupted in the mutant protein; that study is ongoing and will eventually characterize the more than 40 distinct mutations that have been defined in tyrosinase-negative albino patients. The stabilizing interactions of these tyrosinase-related gene products have also been characterized, and many of the mutations involved at those gene loci may affect the stability of this melanogenic complex and its resistance to degradation in the melanosome *in vivo*. We (with Kameyama) have also continued our studies aimed at characterizing a low molecular weight inhibitor of pigment production; this specific inhibitor seems to play an important role in human and murine pigmentation, both with respect to modulating baseline levels of melanogenesis in the cells, but perhaps more importantly, in their rapid responses to stimulation of differentiation. We (with Prota) have continued our characterization of the structure and function of the melanin produced and have made several novel findings about the incorporation of carboxylated precursors into biological melanins. Our studies are now targeted at the further elucidation of the structure of mammalian melanins and how this can be modified by expression of these various gene products, and perhaps most importantly, how that structure might affect the various functional properties of melanins (such as their photoprotective benefit) and their cytotoxic implications to the melanocyte. These problems are being addressed by modifying the expression of the various genes using transfection and antisense approaches, followed by characterization of the melanins and melanosomes formed *in vivo*.

A CRADA (CACR-0159) entitled "Functional Properties of Melanogenic Intermediates" has been established with the KAO CORPORATION (Tochigi, Japan) in March, 1993; that project will begin assessing the implications of the synthesis of different melanogenic precursors on the properties of the melanins produced, especially those related to the photoprotective qualities of melanin as a sunscreen.

2. Melanoma Biology and Immunology. Our studies of antigens specifically expressed by melanoma cells that are involved with immune responses of the host have continued to emphasize the importance of the B700 antigen in those responses. The B700 antigen has been shown to be related to a normal melanocyte-specific constituent, although the metabolic lesion responsible for its production only by transformed cells has thus far remained elusive. Nevertheless, an analogous melanoma specific antigen, termed M66, is also expressed by human melanoma cells and we (with Vlock) have recently found that the murine B700 and the human M66 antigen are quite similar, though not identical, and that several of our respective antibodies are cross-reactive between those antigens showing that they share at least several distinct epitopes. We have shown that melanoma-bearing mice produce specific complement-dependent cytotoxic antibodies which have the same specificity elicited by immunization with irradiated cells (or cell extracts). In our latest studies aimed at examining the efficacy of potential vaccines against melanoma, we (with Shrayer) have repeatedly found that B700 is the immunodominant antigen eliciting beneficial antitumor effects. This protective effect of the vaccine can be further potentiated by concurrent treatment with IL-2. We have successfully developed a murine monoclonal antibody which specifically recognizes B700 and which cross-reacts with a wide variety of types of human melanomas (>95%) and some pigmented nevi (a premalignant state of melanocyte development), but does not cross-react with any other type of nonmelanoma tumor tested or with normal skin. This antibody is now being developed by Centocor through license with NIH as a potential immunodiagnostic tool. In view of the specificity of this antibody to melanoma cells, and the fact that it can provide partial protection to the host against metastatic spread of the tumor, future studies will assess its potential immunotherapeutic use following conjugation to various toxins. Yet another melanoma antigen that represents an altered form of a normal melanocyte constituent, termed B50, has been identified; it is related to a family which includes calcium binding proteins and the Ro/SS-A antigen found in systemic lupus erythematosus. The expression of this antigen is not as highly restricted as is that of B700, and a monoclonal antibody has now been generated against B50 that should prove useful to its further characterization. We (with Gottesman) have begun studies on assessing the roles of various surface and secreted proteases (including urokinase-type plasminogen activator and cathepsins B and L) in the metastatic process by generating a series of sublines of murine melanoma which differ in various phenotypic properties, including their state of differentiation and their metastatic potential. Assessment of the expression of various proteases by those various sublines has demonstrated no definitive correlation of any of the proteases examined with the metastatic potential of the cell. Further studies to assess whether there is a threshold value of any protease required for metastasis will be addressed using the strategy of gene knockout technology.

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- Shrayer D, Kones J, Kouttab N, Bogaars H, Hearing VJ, Gersten DM, Maizel A, Wanebo H. Preimmunization of mice with formalinized extracellular antigens of melanoma in combination with IL-2 and surgical resection increased survival and tumor control in metastatic melanoma model, *J Surg Oncol* 1993;52:142-49.
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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 09101-01 LCBGY
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Analysis of Complexes Between Zinc Finger Proteins and DNA or RNA of HIV-1 Virion		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E. Appella	Medical Officer LCB, NCI
Other:	K. Sakaguchi	Visiting Associate LCB, NCI
	N. Zambrano	Visiting Fellow LCB, NCI
COOPERATING UNITS (if any) A. M. Gronenborn, G. M. Clore, LCP, NIDDK E. T. Baldwin, J. W. Erickson, FCRDC, NCI		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Chemistry		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0.0
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		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Experiments have been done on the structure of a number of proteins involved in HIV transcription, encapsidation and budding. The high resolution three-dimensional structure of a synthetic 57-residue peptide comprising the double zinc finger of the human enhancer binding protein, MBP-1, has been determined by NMR. Each finger comprises an irregular anti-parallel beta-sheet and an alpha-helix, with the zinc tetrahedrally coordinated to two cysteines and two histidines. Comparison of the structure of MBP-1 with the x-ray structure of the Zif-268 triple finger complexed with DNA indicates that the relative orientations of the individual zinc fingers are distinct. Peptides of 39 and 55 residues containing the two zinc binding domains of the p7 nucleocapsid protein have been characterized by physicochemical and nucleic acid binding analyses. Fluorescence and circular dichroism studies demonstrated that the unstructured apo-peptides assume an ordered conformation in the presence of zinc. Computational analysis of a region near the 5' end of the HIV-1 genome revealed a pair of stem-loops separated by 9 and 13 bases. This structure is the binding site of the p7 nucleocapsid protein. Recently, separate studies indicated strong cellular immunity against HIV-1. Cytotoxic T-cells that recognize peptide fragments bound to major histocompatibility complex (MHC) Class I molecules have been identified. The sequence analysis of these peptides should permit the identification of viral epitopes capable of eliciting cellular cytotoxic responses and prove valuable in the development of a vaccine.</p>		

Major Findings:

## a) Structural analysis of a zinc finger of the HIV-1 enhancer binding protein MBP-1.

Previously, we have reported that a peptide of 57 amino acids derived from MBP-1 specifically binds to the HIV-1 enhancer sequence in the 5' viral LTR. This peptide contains two zinc fingers of the type Cys2 His2. We have determined the three dimensional structure of the 57-residue peptide by NMR spectroscopy and each finger is comprised of an irregular anti-parallel  $\beta$ -sheet, hairpin and a single  $\alpha$  helix, with the zinc tetrahedrally coordinated to two cysteines and two histidines. An important feature of both fingers is the presence of a highly packed hydrophobic core comprised of five amino acids in each finger, which serves to stabilize the structure by holding the helix against the  $\beta$ -sheet. This structural characteristic is important functionally and common to a number of DNA binding proteins. A comparison of the double zinc finger solution structure of MBP-1 with the x-ray crystallographic structure of a triple zinc finger (Zif-268) complexed with a DNA oligonucleotide indicates that the relative orientation of the individual fingers is different. This implies that MBP-1 may bind to its DNA in a mode different from that observed for Zif-268. Structural studies of the MBP-1 complexed with an oligonucleotide from the 5' HIV-1 LTR are in progress and should provide information on the interaction of the zinc fingers of MBP-1 with DNA.

## b) Structural characterization of the HIV-1 p7 nucleocapsid protein.

It is known that in HIV-1 the packaging of viral RNA is linked to a dimerization of two identical genomic RNA molecules. Several studies have indicated that the product of the gag precursor protein, p7, is involved in the RNA dimerization. The p7 protein contains two zinc fingers of the type Cys3 His1, and mutations in the cysteine residues interfere with the viral packaging. We have analyzed the physicochemical and nucleic acid binding properties of p7. The two fingers appear as flexible polypeptides that are independently folded. The peptide backbone is folded in a unique conformation compared to the folding of the Cys2 His2 fingers analyzed in the enhancer binding proteins. The fingers from p7 consist of a  $\beta$ -hairpin-like structure containing the two binding cysteines, followed by a loop which is connected to the zinc atom by the histidine and the last cysteine side chain. The two fingers are separated by a very flexible linker segment which contains four basic amino acid residues.

Studies of the tertiary structure of the p7 complexed with an RNA fragment to which it specifically binds have been completed. An interesting finding is the observation that when the sequences of nine different isolates of HIV-1 were subjected to RNA secondary structure analysis, a pattern of two stem-loops in the packaging region ( $\psi$ ) was found. The first stem-loop contains an unpaired A and the 5' major splice junction. To assess the role of this stem-loop structure in the binding specificity of p7, we have estimated binding by gel mobility shift assay and analytical ultracentrifugation. The binding of p7 to a labeled probe containing 44 nucleotides of the  $\psi$  region elicits two complexes showing two bands of different mobility on native gels. The faster migrating complex contained one RNA and one peptide, while the slower migrating one is comprised of two RNA molecules and one peptide as detected by ultracentrifugation. The binding sites of p7 were mapped using RNA derivatives with mutations in different portions of the stem and loop structure. Mutants with base changes that weakened the stem structure of the stem-loop or changed the composition of loop 1 showed complex formation with intermediate mobility and stoichiometry, indicating that the RNA structure plays a role in binding or that the sequence per se directs the binding. In order to further analyze the structural characteristics of the binding site, we have chemically synthesized the single stem-loop 1. Mobility shift analyses showed one major band, suggesting that in this simplified structure the binding is less complex than that of the interaction of p7 with the RNA containing the two stem and loop regions. This complex is comprised of two RNA molecules and one peptide. These data indicate that the sequence of stem-loop 1 plays a role in the binding of p7 and the ensuing dimerization of the RNA molecules. Spontaneous dimerization of the RNA does occur, but this is a slow and inefficient process;

the nucleocapsid protein, p7, is needed to trigger a change in the conformation of the RNA that favors the formation of the dimer or to drive the monomer-dimer equilibrium toward dimer formation. This process of dimerization of the retroviral RNA, aided by the p7 protein and/or gag precursor molecules, is important to repress the 35S RNA translation and facilitate formation of a core chromatin-like structure essential for infectivity. Studies of the tertiary structure of p7 complexed with an RNA fragment to which it specifically binds will aid in the design of antiviral agents capable of inhibiting HIV-1 p7 functions and genomic RNA packaging.

c) Analysis of naturally-processed peptides bound to MHC Class I in HIV-1 infected cells.

Human immunodeficiency virus type-1 (HIV-1) infection is heavily investigated in many laboratories in terms of its immunological reactivity. Cytotoxic T lymphocytes (CTL) specific for HIV-1 may be an important component of the immune response to HIV infection. CTL generally recognize foreign antigens in the form of peptides bound to Class I MHC molecules on the surface of target (e.g., virally-infected) cells. Our goal is to analyze the HIV-1-derived peptides which are naturally processed and presented by Class I molecules in HIV-infected cells expressing various Class I alleles. Currently, a monocytic-cell line, U937, which expresses the HLA-A3.2 Class I molecule, is being analyzed to characterize the peptides normally bound to this molecule. A stable derivative of U937 infected with HIV-1 is available, and preliminary data indicate that the level of A3.2 expression on these cells is equivalent to that of uninfected cells. We will employ the technique of HPLC-electrospray ionization/triple quadrupole mass spectrometry to do a comparative analysis of the peptides bound to Class I molecules in infected vs. uninfected U937 cells. This analysis is currently underway and could have significant implications for the development of a vaccine against HIV-1.

#### Publications

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Sakaguchi K, Zambrano N, Baldwin ET, Shapiro BA, Erickson JW, Omichinski JG, Clore GM, Gronenborn AM, Appella E. Identification of a binding site for the human immunodeficiency virus type 1 nucleocapsid protein, *Proc Natl Acad Sci USA* 1993;in press.

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## SUMMARY STATEMENT

### ANNUAL REPORT LABORATORY OF CELLULAR ONCOLOGY DCBDC, NCI October 1, 1992 through September 30, 1993

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, which have been analyzed by examining proteins that influence the activity of Ras protein. Schwannoma cell lines from patients with neurofibromatosis had low levels of the NF1 product neurofibromin, which correlated with their containing high levels of GTP•Ras. These results were consistent with NF1 being a tumor suppressor gene whose encoded GTPase stimulation negatively regulates Ras. We have also identified neuroblastoma and melanoma cell lines with genetic abnormalities of NF1 and reduced to absent levels of neurofibromin, suggesting that NF1 is acting as a tumor suppressor gene in these cell lines. In contrast to the schwannoma lines, the level of GTP•Ras was low in all lines and did not correlate with that of neurofibromin. These results suggested that NF1 might inhibit cell growth by a mechanism independent of its GTPase stimulatory activity. To confirm this hypothesis, a full length NF1 cDNA was introduced into NIH 3T3 cells. The cells that overexpressed neurofibromin grow more slowly and had normal levels of GTP•Ras. Introduction of the NF1 cDNA into melanoma lines slowed their growth and induced a differentiated phenotype, including an increase in cell size, dendrite formation, and an increase in tyrosinase.

We have also identified four types (I-IV) of apparently full-length cDNAs from a gene CDC25Mm that encodes a ras-specific exchange factor. All four types of cDNAs induced morphologic transformation of NIH 3T3 cells and an increase in the basal level of GTP•Ras. Analysis of expressing ras mutants in these cells indicated that the serum-dependent increase in GTP•Ras by CDC25Mm or by endogenous exchange factors requires membrane association of both Ras and the exchange factor. Morphological transformation of NIH 3T3 cells was observed following co-expression the amino terminus of GAP (GAP-N) v-src (MDSRC) lacking the membrane-localizing sequence. Further analysis suggested that tyrosine phosphorylation and complex formation involving GAP represent critical elements of cell transformation by v-src and that complementation of the cytosolic v-src mutant by GAP-N results, at least in part, from the formation of these complexes.

#### Analysis of Papillomaviruses

Papillomaviruses (PVs) generally induce benign epithelial proliferation at the site of infection. However, there is a strong association between malignant progression of human genital lesions and certain "high risk" HPV types, most frequently HPV16.

Analysis of the structural and immunogenic features of PVs has been hampered by the inability to propagate the viruses in cultured cells. To partially overcome this handicap, we have expressed the L1 major capsid proteins of several human and animal PV types via baculovirus

vectors. The L1 proteins were expressed at high levels and assembled into PV virion-like structures. We have identified two HPV16 L1 clones from primary lesions that, unlike the prototype L1 used in previous studies, efficiently assemble into particles. The self assembled BPV L1 resembled intact virions in being able to induce high titer neutralizing antiserum. These results indicate that L1 has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions. Particles containing both L1 and the L2 minor capsid protein have also been generated. These types of particles might be considered as a candidate for a vaccine to prevent PV infection.

There exists no effective serological assay to measure high risk genital HPV infection. We have developed an ELISA based on HPV16 L1/L2 particles and determined that two-thirds of women who are positive for HPV16 DNA by PCR have significant reactivity to the assembled virion proteins. Less than 10% of the sera from women negative for HPV DNA or positive for low risk HPV6 or HPV11 DNA gave positive reactions. This assay, or a similar one based on a mixture of high risk HPV particles, may aid in determining the natural history of high risk HPV infection and might be useful as an adjunct to Pap screening to identify women at risk for developing cervical cancer.

We have previously reported that BPV E5 induces the ligand independent activation of growth factor receptors. Analysis of receptor chimeras has determined that the transmembrane domain of PDGFR is primarily responsible for its association with and responsiveness to E5, while the intracellular domain of EGFR is required for its association with and activation by E5. These results strongly suggest that E5 forms a complex with an intracellular alpha adaptin-like molecule with strong associated kinase activity. Since E5 inhibits the normal down regulation of EGFR and alpha adaptins are involved in endocytosis of other types of cell surface receptors, it is likely that this association is critical to E5's ability to activate EGFR.

#### Role of protein kinases in modulating cell growth and malignant transformation

The aim of this project is to better understand the role of specific protein kinases in cell growth regulation, malignant transformation, and cellular resistance to chemotherapeutic drugs. Multidrug resistant MCF-7/ADR cells exhibit elevated levels of a modified form of protein kinase C (PKC)-alpha. This altered form of PKC-alpha in the drug resistant MCF-7/ADR cells was highly sensitive to oxidative inactivation when these cells were treated with hydrogen peroxide. In contrast, the PKC-alpha present in drug sensitive MCF-7/WT cells was not altered by hydrogen peroxide treatment. These results indicate that changes in the redox state of the cells, and thus in the oxidative modification of PKC-alpha, may play an important role in modulating the levels of PKC activity found in the drug sensitive versus drug resistant cells.

Treatment of serum-deprived cortical astrocytes with low (subnanomolar) concentrations of vasoactive intestinal peptide (VIP) were observed to induce a significant increase in the level of PKC-alpha in the nuclear fraction. These data further suggest an important role for PKC in the transmission of signals from the plasma membrane to the nucleus, and indicate that very low concentrations of VIP apparently can act through a high affinity binding site which is coupled to a PKC-related signalling pathway.

To better determine if the different isoforms of PKC might possess different regulatory properties and functions within the cell, studies were carried out with stable transfectant NIH 3T3 cells which overexpress either PKC-delta or PKC-epsilon. It was found that overproduction of either of these PKC isoforms resulted in increased sodium-dependent phosphate uptake, but through apparently different mechanisms. These findings suggest that different PKC isozymes may act distinctly and specifically to regulate an in vivo cellular process.

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

PROJECT NUMBER  
 Z01 CB 03663-17 LCO

PERIOD COVERED

October 1, 1992 through September 30, 1993

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,

PI:	D. R. Lowy	Chief, LCO	LCO, NCI
OTHER:	J. E. DeClue	IRTA Fellow	LCO, NCI
	H. Cen	Visiting Associate	LCO, NCI
	M. Johnson	IRTA Fellow	LCO, NCI
	A. Konig	Special Volunteer	LCO, NCI
	N. Tayebi	Predoctoral IRTA Fellow	LCO, NCI
	A. G. Papageorge	Microbiologist	LCO, NCI
	W. C. Vass	Biologist	LCO, NCI

COOPERATING UNITS (if any)

University Microbiology Institute, Copenhagen, Denmark, Dr. B. Willumsen  
 Hebrew University of Jerusalem, Jerusalem, Israel, Dr. A. Levitzki  
 Department of Genetics, University of Utah School of Medicine, Dr. R. White

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

TOTAL STAFF YEARS:

8.0

PROFESSIONAL:

6.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

(a) Human       (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, which have been analyzed by examining proteins that influence the activity of Ras protein. Schwannoma cell lines from patients with neurofibromatosis had low levels of the NF1 product neurofibromin, which correlated with their containing high levels of GTP•Ras. These results were consistent with NF1 being a tumor suppressor gene whose encoded GTPase stimulation negatively regulates Ras. We have also identified neuroblastoma and melanoma cell lines with genetic abnormalities of NF1 and reduced to absent levels of neurofibromin, suggesting that NF1 is acting as a tumor suppressor gene in these cell lines. In contrast to the schwannoma lines, the level of GTP•Ras was low in all lines and did not correlate with that of neurofibromin. These results suggested that NF1 might inhibit cell growth by a mechanism independent of its GTPase stimulatory activity. To confirm this hypothesis, a full length NF1 cDNA was introduced into NIH 3T3 cells. The cells that overexpressed neurofibromin grow more slowly and had normal levels of GTP•Ras. Introduction of the NF1 cDNA into melanoma lines slowed their growth and induced a differentiated phenotype, including an increase in cell size, dendrite formation, and an increase in tyrosinase.

We have also identified four types (I-IV) of apparently full-length cDNAs from a gene CDC25Mm that encodes a ras-specific exchange factor. All four types of cDNAs induced morphologic transformation of NIH 3T3 cells and an increase in the basal level of GTP•Ras. Analysis of expressing ras mutants in these cells indicated that the serum-dependent increase in GTP•Ras by CDC25Mm or by endogenous exchange factors requires membrane association of both Ras and the exchange factor. Morphological transformation of NIH 3T3 cells was observed following co-expression the amino terminus of GAP (GAP-N) v-src (MDSRC) lacking the membrane-localizing sequence. Further analysis suggested that tyrosine phosphorylation and complex formation involving GAP represent critical elements of cell transformation by v-src and that complementation of the cytosolic v-src mutant by GAP-N results, at least in part, from the formation of these complexes.

Major findings:

1. ras oncogenes. Normal ras proto-oncogene function is required for growth factor mediated mitogenesis, and mutationally activated ras genes have been identified in a variety of human and animal tumors. We have been studying ras function by examining proteins that influence the activity of Ras protein. We have examined the influence of three proteins on ras - GAP, NF1, and an CDC25Mm. GAP is a protein that can, via its GTPase accelerating activity, inactivate normal Ras protein; highly transforming versions of Ras protein are resistant to this activity. In addition to being a negative regulator of Ras, GAP is also a candidate for being a Ras target in higher eukaryotes. NF1 is the gene that is mutated in patients with type 1 neurofibromatosis. NF1 possesses a GAP-like catalytic activity against Ras protein and shares significant homology with negative regulators of yeast Ras. CDC25Mm is a Ras-specific guanine nucleotide exchange factor that activates Ras.

Schwannoma cell lines from patients with neurofibromatosis had low levels of the NF1 product neurofibromin, which correlated with their containing high levels of GTP•Ras. These results were consistent with NF1 being a tumor suppressor gene whose encoded GTPase stimulation negatively regulates Ras. We have also identified neuroblastoma and melanoma cell lines with genetic abnormalities of NF1 and reduced to absent levels of neurofibromin, suggesting that NF1 is acting as a tumor suppressor gene in these cell lines. In contrast to the schwannoma lines, the level of GTP•Ras was low in all lines and did not correlate with that of neurofibromin. These results suggested that NF1 might inhibit cell growth by a mechanism independent of its GTPase stimulatory activity. To confirm this hypothesis, a full length NF1 cDNA was introduced into NIH 3T3 cells. The cells that overexpressed neurofibromin grow more slowly and had normal levels of GTP•Ras. Introduction of the NF1 cDNA into melanoma lines slowed their growth and induced a differentiated phenotype, including an increase in cell size, dendrite formation, and an increase in tyrosinase.

We recently identified four types (I-IV) of apparently full-length cDNAs from CDC25Mm. The largest cDNA (type IV) is brain-specific, with the other three classes, although they have distinct 5' ends, essentially representing progressive N-terminal deletions of this cDNA. All four types of cDNAs induced morphologic transformation of NIH 3T3 cells and an increase in the basal level of GTP•Ras. Serum stimulation of these transformants lead to a further increase in GTP•Ras only in cells expressing the largest cDNA. Each type of CDC25Mm protein was found in cytosolic and membrane fractions. Analysis of expressing ras mutants in these cells indicated that the serum-dependent increase in GTP•Ras by CDC25Mm or by endogenous exchange factors requires membrane association of both Ras and the exchange factor.

Morphological transformation of NIH 3T3 cells was observed following co-expression the amino terminus of GAP (GAP-N) v-src (MDSRC) lacking the membrane-localizing sequence. Cells expressing either of these genes alone remained nontransformed. For transformation induced by wild-type v-src as well as by co-expression of MDSRC and GAP-N, a strict correlation was observed between cell transformation and complex formation involving GAP and the tyrosine phosphorylated proteins p62, p190, and a novel protein of 150 kd. As with cells transformed by wild-type v-src, the MDSRC plus GAP-N transformants remained dependent on endogenous Ras. The results suggest that tyrosine phosphorylation and complex formation involving GAP represent critical elements of cell transformation by v-src and that complementation of the cytosolic v-src mutant by GAP-N results, at least in part, from the formation of these complexes.

Publications:

- Zhang K, Papageorge AG, Lowy, DR. Mechanistic aspects of signaling through Ras in NIH 3T3 cells. *Science* 1992;257:671-4.
- Cen H, Papageorge AG, Zippel R, Lowy DR, Zhang K. Isolation of multiple mouse cDNAs with coding homology to *Saccharomyces cerevisiae* CDC25: identification of a region related to Bcr, Vav, Dbp, and CDC24. *EMBO J* 1992;11:4007-15.
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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
 Z01 CB 08905-12 LCO

PERIOD COVERED  
 October 1, 1992 through September 30, 1993

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,

PI:	W. B. Anderson	Research Chemist	LCO NCI
OTHER:	Z. Olah	Visiting Associate	LCO NCI
	J. W. Karaszkiwicz	Biotechnology Fellow	LCO NCI
	C. Lehel	Visiting Fellow	LCO NCI
	A. Ferrier	IRTA Fellow	LCO NCI
	J. A. Strasburger	Microbiologist	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 STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	4.5	0.5

CHECK APPROPRIATE BOX(ES)

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

B

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

The aim of this project is to better understand the role of specific protein kinases in cell growth regulation, malignant transformation, and cellular resistance to chemotherapeutic drugs. Multidrug resistant MCF-7/ADR cells exhibit elevated levels of a modified form of protein kinase C (PKC)-alpha. This altered form of PKC-alpha in the drug resistant MCF-7/ADR cells was highly sensitive to oxidative inactivation when these cells were treated with hydrogen peroxide. In contrast, the PKC-alpha present in drug sensitive MCF-7/WT cells was not altered by hydrogen peroxide treatment. These results indicate that changes in the redox state of the cells, and thus in the oxidative modification of PKC-alpha, may play an important role in modulating the levels of PKC activity found in the drug sensitive versus drug resistant cells.

Treatment of serum-deprived cortical astrocytes with low (subnanomolar) concentrations of vasoactive intestinal peptide (VIP) were observed to induce a significant increase in the level of PKC-alpha in the nuclear fraction. These data further suggest an important role for PKC in the transmission of signals from the plasma membrane to the nucleus, and indicate that very low concentrations of VIP apparently can act through a high affinity binding site which is coupled to a PKC-related signalling pathway. To better determine if the different isoforms of PKC might possess different regulatory properties within the cell, studies were carried out with stable transfectant NIH 3T3 cells which overexpress either PKC-delta or PKC-epsilon. Overproduction of either PKC isoform resulted in increased sodium-dependent phosphate uptake, but through apparently different mechanisms. These findings suggest that different PKC isozymes may act distinctly and specifically to regulate an in vivo cellular process.

Previously, it was shown that the RI and RII regulatory subunits of cAMP-dependent protein kinase (PKA) can be covalently modified by retinoylation. Results indicate that the RI and RII subunits also are retinoylated in human fibroblasts, and that the level of [3-H] RA labeling (retinoylation) of the RII subunit was greater in psoriatic fibroblasts than in normal fibroblasts. These results suggest that retinoylation may be involved, at least in part, in the reversal of the decreased levels of PKA activities observed when psoriatic cells are treated with RA.

Cooperating Units:

Laboratory of Genetics, NCI, NIH, Dr. J. F. Mushinski  
 Lab of Developmental Neurobiology, NICHD, NIH, Dr. D. E. Brenneman  
 Dept. Pharmacology, USC School of Medicine, Los Angeles, CA, Dr. R. Gopalakrishna  
 Lab of Physiopath. Development, Paris, France, Drs. D. Evain-Brion, and F. Raynaud

Major Findings:

1. Protein Kinase C. Protein kinase C (PKC) is a serine/threonine phosphotransferase which plays a crucial role in transmembrane signal transmission. Evidence indicates that PKC also is involved in modulating cellular resistance to antitumor drugs of the natural products class. Previously, we demonstrated that multidrug-resistant human breast carcinoma MCF-7/ADR cells have increased levels of PKC-alpha compared to drug-sensitive MCF-7/WT cells, with a significantly elevated pool of PKC-alpha found in the nucleus of the MCF-7/ADR cells. Since others have reported that multidrug resistant cells exhibit altered levels of components of cellular redox pathways, and since we have shown that oxidative modification may be an important regulatory parameter of PKC, studies were carried out to assess changes in the susceptibility of PKC present in different subcellular fractions to oxidation in response to treatment of MCF-7/WT and MCF-7/ADR cells with hydrogen peroxide. The elevated pools of PKC-alpha present in the nuclear and cytosolic fractions of MCF-7/ADR cells were found to be highly sensitive to inactivation and subsequent down-regulation with short-term exposure of these cells to hydrogen peroxide. No change was noted in PKC levels when MDV-7/WT cells were treated under similar conditions with hydrogen peroxide. These results indicate that drug-resistant MDF-7/ADR cells contain an apparently modified form of PKC-alpha which is highly sensitive to oxidative inactivation, and that the increased oxygen radical scavenging capacity present in the MCF-7/ADR cells may protect the nuclear pool of this form of PKC-alpha found in these drug resistant cells.

Other studies were carried out to determine if PKC might be involved in the mitogenic signal transduction mechanisms elicited by vasoactive intestinal peptide (VIP) treatment of rat neonatal cortical astrocytes. Treatment of serum-deprived astrocytes in culture with low (0.1nM) concentrations of VIP was observed to cause a significant increase in the level of PKC-alpha found at the nucleus. These results suggest that PKC-alpha may be involved in the signal transduction process activated by VIP binding to high affinity receptors. The change in nuclear localization of PKC-alpha in response to subnanomolar VIP may be involved in mediating the cellular response(s) to this peptide during neurodevelopment.

Protein kinase C consists of a family of related isozymes. To determine if the different PKC isozymes might have distinct regulatory functions within the cell, studies were carried out with stable transfectant NIH 3T3 cells which overexpressed either PCK-delta or PKC-epsilon. Previously it was found that activation of PKC in turn caused the rapid stimulation of sodium-dependent phosphate transport. A change in the level of phosphate uptake thus was utilized as a cellular marker to detect the biological effects of different forms of PKC within intact cells. Overexpression of both PKC-delta and PKC-epsilon resulted in elevated levels of sodium-dependent phosphate uptake. However, overexpression of PKC-delta caused an increase in the  $V_{max}$  of Na/Pi uptake, while overexpression of PKC-epsilon resulted in an increase in the  $K_m$  for orthophosphate. These findings suggest that each of the PKC isotypes investigated may contribute to the regulation of phosphate uptake, but through apparently distinct mechanisms.

2. Retinoids in mediating cell growth and tumor promotion. Studies were continued, in collaboration with Dr. D. Evain-Brion, to better understand the reason for the decreased levels of cyclic AMP-dependent protein kinase (PKA), cyclic AMP binding, and phosphotransferase

activities in the hyperproliferative skin disease psoriasis, and to study the possible mechanism(s) by which retinoic acid (RA) treatment of psoriatic cells increases the low levels of PKA activities toward normal. Previously, in studies with Dr. T. Breitman, it was shown that the RII regulatory subunit can be covalently modified by retinoylation (formation of a thioester bond between cysteine residues and RA). Thus, studies were carried out to determine possible differences in the extent of retinoylation of the RI and RII subunits of normal and psoriatic cells. It was observed that the RII subunit was covalently labelled with [<sup>3</sup>H]RA (retinoylated) to a greater extent in psoriatic fibroblasts compared to normal fibroblasts. No significant difference was detected in the level of retinoylation of the RI subunit between normal and psoriatic cells. These results suggest that RA may act to enhance cyclic AMP binding and PKA activity in psoriatic cells through covalent modification (retinoylation) of the regulatory subunits of PKA.

Publications:

Raynaud F, Gerbaud P, Bouloc A, Gorin I, Anderson WB, Evain-Brion D. Rapid effect of the synthetic retinoid acitretin on psoriatic erythrocytes to increase 8-azido cyclic AMP binding to the RI regulatory subunit. *J Invest Dermatol* 1993; 100:77-81.

Gundimeda U, Hara SK, Anderson WB, Gopalakrishna R. Retinoids inhibit the oxidative modification of protein kinase C induced by oxidant tumor promoters. *Archives Biochem Biophys* 1993;300:526-30.

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

PROJECT NUMBER

Z01 CB 09052-05 LCO

PERIOD COVERED

October 1, 1992 through September 30, 1993

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,

PI:	J. T. Schiller	Research Microbiologist	LCO NCI
OTHER:	D. R. Lowy	Chief, LCO	LCO NCI
	B. D. Cohen	IRTA Fellow	LCO NCI
	R. Kirnbauer	Visiting Fellow	LCO NCI
	R. M. Melillo	Visiting Fellow	LCO NCI
	R. B. Roden	Visiting Fellow	LCO NCI
	N. L. Hubbert	Microbiologist	LCO NCI
	J. V. Taub	Bio Lab Technician	LCO NCI

COOPERATING UNITS (if any)

Lab of Structural Biology Research, NIAMS, NIH, Drs. F. Booy and N. Cheng

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

TOTAL STAFF YEARS:

7.0

PROFESSIONAL:

5.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

B

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

Papillomaviruses (PVs) generally induce benign epithelial proliferation at the site of infection. However, there is a strong association between malignant progression of human genital lesions and certain "high risk" HPV types, most frequently HPV16.

Analysis of the structural and immunogenic features of PVs has been hampered by the inability to propagate the viruses in cultured cells. To partially overcome this handicap, we have expressed the L1 major capsid proteins of several human and animal PV types via baculovirus vectors. The L1 proteins were expressed at high levels and assembled into PV virion-like structures. We have identified two HPV16 L1 clones from primary lesions that, unlike the prototype L1 used in previous studies, efficiently assembles into particles. The self assembled BPV L1 resembled intact virions in being able to induce high titer neutralizing antiserum. These results indicate that L1 has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions. Particles containing both L1 and the L2 minor capsid protein have also been generated. These types of particles might be considered as a candidate for a vaccine to prevent PV infection.

There exists no effective serological assay to measure high risk genital HPV infection. We have developed an ELISA based on HPV16 L1/L2 particles and determined that two-thirds of women who are positive for HPV16 DNA by PCR have significant reactivity to the assembled virion proteins. Less than 10% of the sera from women negative for HPV DNA or positive for low risk HPV6 or HPV11 DNA gave positive reactions. This assay, or a similar one based on a mixture of high risk HPV particles, may aid in determining the natural history of high risk HPV infection and might be useful as an adjunct to Pap screening to identify women at risk for developing cervical cancer.

We have previously reported that BPV E5 induces the ligand independent activation of growth factor receptors. Analysis of receptor chimeras has determined that the transmembrane domain of PDGFR is primarily responsible for its association with and responsiveness to E5, while the intracellular domain of EGFR is required for its association with and activation by E5. These results strongly suggest that E5 activates the two receptors by different mechanisms.

Major Findings:

Background: Papillomaviruses (PVs) infect the epithelia of a wide variety of animals and humans. They generally induce benign proliferation at the site of infection, but lesions induced by certain PVs undergo malignant progression. There is a strong association between malignant progression of human genital lesions and certain "high risk" HPV types, most frequently HPV16. The major goals of the laboratory have been to elucidate the mechanisms by which the viral oncoproteins induce transformation, develop a method to identify people at high risk for developing cervical and other genital cancers by assays that detect infection with high risk HPV, and generate immunogens for development of a safe and effective vaccine to prevent genital HPV infection.

1. Self-assembly of the PV L1 and L1 plus L2 into particles that are morphologically and immunologically similar to native virions. Analysis of the structural and immunogenic features of papillomavirus virions has been hampered by the inability to propagate the viruses in cultured cells. To partially overcome this handicap, we have expressed the L1 major capsid protein of HPV6, HPV11, HPV16, HPV31, BPV1, RhPV, and CRPV in insect cells via baculovirus vectors. The L1 proteins were expressed at high levels and assembled into structures that closely resembled PV virions. In contrast to bacterially derived L1, the self-assembled L1 mimicked intact BPV virions in being able to induce high titer neutralizing rabbit antisera. These results indicate that L1 protein has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions. Unlike other PV L1s, the widely used prototype HPV16 L1 did not efficiently self-assemble. We therefore tested two L1 genes that were isolated from less progressed lesions and found that both of them encode L1 proteins that efficiently self-assemble. A single amino acid difference accounts for the difference in assembly potential. We have also co-expressed L1 and the L2 minor capsid protein of several human and animal types via doubly recombinant baculoviruses and have determined that L2 is incorporated into the particles. These types of particles might be considered as a candidate reagents for a serological test to measure the prevalence of neutralizing antibodies and for a vaccine to prevent PV infection.

2. A virus-like particle ELISA detects serum antibodies in a majority of women infected with HPV16. Previous serological assays to identify high risk HPV infection have either not correlated with other indicators of HPV infection or identified only a minority of infected individuals. However, properly folded virion proteins or native virions have not been available for testing. We have developed an ELISA using HPV16 L1 plus L2 particles as the antigen. The sera of two-thirds of women positive for genital HPV16 DNA by PCR had reactivities that were significantly greater than the mean reactivity of the sera from women who tested negative for HPV DNA. By the same criteria, 6% of women negative for HPV DNA and 9% of those positive for low risk HPV6 or HPV11 DNA were positive in the ELISA. Among the HPV16 DNA positives, the highest percentage of ELISA positivity (75%) was found in the sera from women with severe cervical dysplasia, while the lowest percentage (42%) was found in sera from women who tested positive for HPV16 DNA by PCR but not by a less sensitive method and were cytologically normal. This suggests that the ELISA may be a relatively good measure of clinically significant HPV16 infection. A similar assay, using a mixture of high risk HPV particles as antigen, might aid in determining the natural history of high risk HPV infection and perhaps help to identify women at risk for developing cervical cancer.

3. The E5 responsive domains of PDGF and EGF receptors. We have previously shown that E5 induces the ligand independent activation of growth factor receptors. To determine the domains responsible for E5 induced activation, we have made a series of chimeras between PDGFR and EGFR, separating the molecules into extracellular, transmembrane, and intracellular domains. Analysis of the chimeras indicated that E5 activation of the PDGFR specifically requires the PDGFR transmembrane domain, and activation of EGFR specifically requires the EGFR

intracellular domain. E5 was found in a complex with both receptors and the domain required for this association was the same as the domain required for activation. These results imply that E5 activates the two receptors by relatively direct but distinct mechanisms.

4. Identification and characterization of an E5 associated cellular protein. The identification of E5 associated cellular proteins has been hampered by its small size (44 amino acids) and high degree of hydrophobicity. To partially overcome this problem, we have expressed the C-terminal domain of E5, which is hydrophilic and required for E5 transforming activity, as a fusion protein linked to bacterial glutathione-S-transferase (GST). The GST-E5 was purified on a glutathione containing column, and the bound protein incubated with cellular extracts. A single 125 Kd cellular protein specifically bound the wild type E5 peptide but not the corresponding peptides with single residue changes in conserved amino acids. This cellular protein, which is associated with a strong in vitro kinase activity, was preparatively purified and subjected to biochemical and partial sequence analysis. It appears to be a new member of the alpha adaptin family. It is likely that the association between p125 and E5 has biological significance, since E5 inhibits the normal down regulation of activated EGFR and alpha adaptins have been implicated in the coated pit mediated endocytosis of another class of cell surface receptors.

5. p53 metabolism in E6 containing cells. It has previously been determined that in vitro synthesized HPV16 E6 binds the tumor suppressor protein p53 and induces its in vitro degradation. Since it has not been determined if E6 influences the metabolism of p53 in vivo, we have measured the half-life of newly synthesized p53 in human keratinocytes before and after the introduction of HPV16 E6. The p53 in normal human keratinocytes had a surprisingly long half-life of 4 hr while the E6 expressing derivatives had half-lives of less than 30 min. In the absence of E7, the E6 expressing cells rapidly senesced indicating that rapid degradation of p53 is not sufficient to induce immortalization. In many lines immortalized by E6 plus E7, the total amount of p53, as measured by immunoblotting, was not significantly reduced. These results suggest that E6 may preferentially degrade newly synthesized p53 and therefore that this pool may be preferentially active in growth suppression.

#### Publications:

Sedman SA, Hubbert NL, Vass WC, Lowy DR, Schiller JT. Mutant p53 can substitute for Human Papillomavirus type 16 E6 in immortalization of human keratinocytes but does not have the E6-associated *trans*-activation or transforming activity. *J Virol* 1992;66:4201-8.

Hubbert NL, Sedman SA, Schiller JT. HPV16 E6 increases the degradation rate of p53 in human keratinocytes. *J Virol* 1992;66:6237-41.

Kimbauer R, Booy F, Cheng N, Lowy DR, Schiller, JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc Natl Acad Sci USA* 1992;89:12180-4.

Kimbauer R, Roden R, Lowy DR, Schiller JT, Booy F, Cheng N. Papillomavirus L1 major capsid protein: self-assembly into particles that are morphologically and immunologically similar to native virions. In: *Vaccines 93*, Cold Spring Harbor Press, 1993; in press.

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Lowy DR, Androphy EJ. Warts. In: Fitzpatrick TB, et al, eds. *Dermatology in General Medicine*, 4th ed. New York: McGraw-Hill, 1993;in press.



Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1992 to September 30, 1993

The genetic basis of human renal cell carcinoma has been the major focus of the research effort of the Cellular Immunity Section. Our major accomplishment this year is the isolation of the von Hippel-Lindau (VHL) tumor suppressor gene. The newly isolated gene appears to play a major role in the pathogenesis of sporadic renal cell carcinoma.

The identification of the VHL tumor suppressor gene is the result of 8 years of study of the genetic basis of human renal cell carcinoma. The cloning of this gene is another example of the power of positional cloning. The VHL gene was isolated after detailed genetic and physical mapping of the VHL region. The identification of 3 nested germ line deletions by pulsed field gel electrophoresis proved critical in the work.

One family has been identified with papillary renal cell carcinoma, an uncommon histologic type of renal cell carcinoma. The disorder was not linked to polymorphic markers on chromosome 3p and there was no loss of heterozygosity at loci on 3p in renal tumors.

While supported by a grant from the National Center for Genome Research, we have assisted in the preparation of a high resolution genetic map of human chromosome 3. During the last years, 30 highly polymorphic microsatellite markers were identified and placed on the genetic map of chromosome 3.

The Immunopathology Section has continued studies on three pro-inflammatory proteins: neutrophil attractant protein-1 (NAP-1, IL-8), monocyte chemoattractant protein-1 (MCP-1), and macrophage stimulating protein (MSP). We previously reported the purification and cloning of human monocyte chemoattractant protein-1 (MCP-1). Then we cloned the cDNA from several species including rabbit, rat, and guinea pig, which was the next step in our plan to study the role of MCP-1 and NAP-1 in vivo. Since the last report, we have expressed recombinant animal MCP-1 and NAP-1 to test their chemotactic activity and to raise antibodies. Several aspects of this work are of interest. [1] The antibodies to rat recombinant MCP-1 specifically immunoprecipitated radiolabeled rat MCP-1 produced by Con A-stimulated rat spleen cells, indicating that the antibodies recognize epitopes of the native protein. It is therefore possible that they will inhibit biological activity. [2] Guinea pig recombinant MCP-1 was a poor attractant for guinea pig peritoneal macrophages, but a potent attractant for human blood monocytes, despite the fact that it has only 57% amino acid sequence similarity to human MCP-1. Intradermal injection of gpMCP-1 caused marked infiltration of monocytes (but not neutrophils) into the injected sites after 4 to 6 hours. These results indicate that MCP-1 causes recruitment of blood monocytes into tissues, whereas it has little effect on tissue macrophages. [3] Although gpNAP-1 has 75% amino acid sequence similarity to human NAP-1, gpNAP-1 was not a good chemoattractant for human neutrophils. However, gpNAP-1 attracted guinea pig neutrophils in vitro, and intradermal injection of gpNAP-1 into guinea pig skin caused marked neutrophil infiltration. The E-L-R sequence that precedes the first

cysteine of human NAP-1 is critical for the biological activity of NAP-1. In addition to the high amino acid sequence similarity of gpNAP-1 to human NAP-1, the E-L-R sequence is completely conserved in gpNAP-1, suggesting that other sequences of NAP-1 are also involved in the expression of the biological activity. [4] Unlike MCP-1, neutrophil attractant protein-1 (NAP-1) has not been identified in rat or mouse. Therefore, these animals are not suitable to study the role of NAP-1 in vivo. In contrast, we succeeded in cloning NAP-1 cDNA from guinea pig spleen cells. These findings suggest that the guinea pig is more suitable than rat or mouse as an animal model. We are now developing antibodies against gpMCP-1 and gpNAP-1 to use guinea pig as a model to study the mechanism of neutrophil and monocyte accumulation into inflammatory or non-inflammatory immune reaction sites.

Macrophage stimulating protein (MSP) was discovered as a biological activity that makes mouse resident peritoneal macrophages capable of a chemotactic response to C5a. Based on partial sequence data from highly purified MSP, we designed a probe that led to cloning of an MSP cDNA from a library of the Hep G2 human hepatoma cell line. Since the open reading frame of the MSP cDNA is identical to that of the exon sequence of a human gene described by Han et al, the gene for MSP is now known, and is located on chromosome 3p. During the past year, we have developed new antibodies to MSP, and have also studied the action and target cell specificity of pure MSP in vitro.

MSP is a disulfide-linked 2-chain protein. It is a member of a family of proteins characterized by a highly conserved triple disulfide loop structure (kringle) that may be repeated up to 7 times in the  $\alpha$ -chain. These proteins are secreted as precursors, which have no biological activity until the single protein chain is cleaved into  $\alpha$  and  $\beta$  chains by specific serine proteases at an arginine-valine bond. The  $\alpha$ - $\beta$  chain junction of MSP is also arginine-valine. We have postulated that by analogy to other members of the family, MSP circulates as biologically inactive pro-MSP. Activation in tissues would occur by proteolytic cleavage of pro-MSP induced by as yet unknown stimuli. We found MSP but no pro-MSP in serum or plasma, probably due to conversion of pro-MSP to MSP by a serine protease of the clotting cascade. We developed antibodies to human MSP for detection in Western blots, quantification in biological fluids, and neutralization of activity. Immunogens included native MSP, reduced and alkylated  $\alpha$  and  $\beta$  chains, and peptides of MSP regions with minimal sequence similarity to other kringle proteins. We obtained three antibody categories, based on interaction with the following types of epitope: [1] primary sequence; [2] discontinuous (dependent on disulfide bonds); and [3] cryptic (not exposed in native MSP). None of the antibodies reacted with related kringle proteins. A specific sandwich ELISA was developed for measurement of human MSP. The mean serum concentration was 4 nM. Serum MSP did not increase over a 24 hour period in response to intravenous LPS, indicating that MSP is not an acute phase protein. These findings are consistent with the hypothesis that regulation of MSP activity is by conversion of pro-MSP to MSP, rather than by rapid changes in rates of synthesis.

MSP induces mouse resident peritoneal macrophages to become responsive to the chemoattractant C5a and to ingest C3bi-coated erythrocytes. We have now shown that MSP action is not limited to complement-induced responses, since it also induced responsiveness to the non-complement chemoattractant, casein. In addition to stimulating responsiveness to attractants, MSP functioned alone as a chemoattractant for resident peritoneal macrophages. A critical difference between MSP and C5a is

that resident macrophages did not migrate to C5a without an additional stimulus such as MSP in the cell suspension, whereas macrophages suspended in medium alone migrated to MSP in the attractant well. Thus, in contrast to C5a, MSP appears capable of a dual role, both activator and attractant. MSP had no effect on responsiveness of mouse peritoneal exudate macrophages to C5a; nor could it attract exudate macrophages or human blood monocytes. Absorption studies showed that resident macrophages have a receptor for MSP, but exudate macrophages do not. In view of these findings, it appears that the biological role of MSP is not as a recruiter of blood monocytes to sites of inflammation, but as an activator of mature macrophages. The MSP-induced activated state for responsiveness to C5a or C3bi was transient, and decayed at a first order rate with a half-time of about 1 hour. This is a new example of the transience of activation induced in macrophages by pro-inflammatory stimuli.

In an exploration of other possible cellular actions of MSP, we studied the effect of MSP on cytokine or LPS-induced nitric oxide (NO) synthesis in mouse macrophages. MSP strongly inhibited LPS-induced NO synthesis in a dose dependent manner. Moreover, NO synthesis induced by IFN- $\gamma$  plus IL-2 or TNF- $\alpha$ , which may use different pathways for inducing NO release, was also blocked by MSP. Northern blot analysis showed that MSP suppressed the expression of NO synthase mRNA in both dose and time-dependent fashions. The inhibition is not non-specific, because the expression of monocyte chemoattractant protein-1 mRNA induced by LPS was not affected.

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

PROJECT NUMBER

Z01 CB 08575-20 LIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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,

PI: E. Leonard Chief, Immunopathology Section, LIB, NCI

Other: T. Yoshimura Visiting Scientist

M.H. Wang Visiting Fellow

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Immunopathology Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, MD 21702

TOTAL STAFF YEARS:

4.0

PROFESSIONAL:

3.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews

B

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

Investigations in the Immunopathology Section are on chemotactic and other immune effector responses of leukocytes. The emphasis is on chemotaxis, a mechanism by which cells are attracted to inflammatory sites, delayed hypersensitivity reactions and growing tumors. The project includes chemistry and biology of chemotactic factors secreted in response to inflammatory stimuli, and characterization of a serum protein that modulates macrophage motility.



The Immunopathology Section has continued studies on three pro-inflammatory proteins: neutrophil attractant protein-1 (NAP-1, IL-8), monocyte chemoattractant protein-1 (MCP-1), and macrophage stimulating protein (MSP). We previously reported the purification and cloning of human monocyte chemoattractant protein-1 (MCP-1). Then we cloned the cDNA from several species including rabbit, rat, and guinea pig, which was the next step in our plan to study the role of MCP-1 and NAP-1 in vivo. Since the last report, we have expressed recombinant animal MCP-1 and NAP-1 to test their chemotactic activity and to raise antibodies. Results are summarized in the accompanying Table.

Protein	Mass (SDS-PAGE)	Sequence similarity to human, %	Attractant activity	Antibodies	
				Polyclonal	Monoclonal
Rat MCP-1	30 kD	52%	4+ to hu monocytes	yes	yes
GP MCP-1	25 kD	57%	4+ to hu monocytes ± to gp macrophages 4+ gp intradermal	in progress	
GP NAP-1	7 kD	75%	± to hu neutrophils 4+ to gp neutrophils 4+ gp intradermal	in progress	

Several aspects of this summary table are of interest. [1] The antibodies to rat recombinant MCP-1 specifically immunoprecipitated radiolabeled rat MCP-1 produced by Con A-stimulated rat spleen cells, indicating that the antibodies recognize epitopes of the native protein. It is therefore possible that they will inhibit biological activity. [2] Guinea pig recombinant MCP-1 was a poor attractant for guinea pig peritoneal macrophages, but a potent attractant for human blood monocytes, despite the fact that it has only 57% amino acid sequence similarity to human MCP-1. Intradermal injection of gpMCP-1 caused marked infiltration of monocytes (but not neutrophils) into the injected sites after 4 to 6 hours. These results indicate that MCP-1 causes recruitment of blood monocytes into tissues, whereas it has little effect on tissue macrophages. [3] Although gpNAP-1 has 75% amino acid sequence similarity to human NAP-1, gpNAP-1 was not a good chemoattractant for human neutrophils. However, gpNAP-1 attracted guinea pig neutrophils in vitro, and intradermal injection of gpNAP-1 into guinea pig skin caused marked neutrophil infiltration. The E-L-R sequence that precedes the first cysteine of human NAP-1 is critical for the biological activity of NAP-1. In addition to the high amino acid sequence similarity of gpNAP-1 to human NAP-1, the E-L-R sequence is completely conserved in gpNAP-1, suggesting that other sequences of NAP-1 are also involved in the expression of the biological activity. [4]

Unlike MCP-1, neutrophil attractant protein-1 (NAP-1) has not been identified in rat or mouse. Therefore, these animals are not suitable to study the role of NAP-1 in vivo. In contrast, we succeeded in cloning NAP-1 cDNA from guinea pig spleen cells. These findings suggest that the guinea pig is more suitable than rat or mouse as an animal model. We are now developing antibodies against gpMCP-1 and gpNAP-1 to use guinea pig as a model to study the mechanism of neutrophil and monocyte accumulation into inflammatory or non-inflammatory immune reaction sites.

Macrophage stimulating protein (MSP) was discovered as a biological activity that makes mouse resident peritoneal macrophages capable of a chemotactic response to C5a. Based on partial sequence data from highly purified MSP, we designed a probe that led to cloning of an MSP cDNA from a library of the Hep G2 human hepatoma cell line. Since the open reading frame of the MSP cDNA is identical to that of the exon sequence of a human gene described by Han et al, the gene for MSP is now known, and is located on chromosome 3p. During the past year, we have developed new antibodies to MSP, and have also studied the action and target cell specificity of pure MSP in vitro.

MSP is a disulfide-linked 2-chain protein. It is a member of a family of proteins characterized by a highly conserved triple disulfide loop structure (kringle) that may be repeated up to 7 times in the  $\alpha$ -chain. These proteins are secreted as precursors, which have no biological activity until the single protein chain is cleaved into  $\alpha$  and  $\beta$  chains by specific serine proteases at an arginine-valine bond. The  $\alpha$ - $\beta$  chain junction of MSP is also arginine-valine. We have postulated that in analogy to other members of the family, MSP circulates as biologically inactive pro-MSP. Activation in tissues would occur by proteolytic cleavage of pro-MSP induced by as yet unknown stimuli. We found MSP but no pro-MSP in serum or plasma, probably due to conversion of pro-MSP to MSP by a serine protease of the clotting cascade. We developed antibodies to human MSP for detection in Western blots, quantification in biological fluids, and neutralization of activity. Immunogens included native MSP, reduced and alkylated  $\alpha$  and  $\beta$  chains, and peptides of MSP regions with minimal sequence similarity to other kringle proteins. We obtained three antibody categories, based on interaction with the following types of epitope: [1] primary sequence; [2] discontinuous (dependent on disulfide bonds); and [3] cryptic (not exposed in native MSP). None of the antibodies reacted with related kringle proteins. A specific sandwich ELISA was developed for measurement of human MSP. The mean serum concentration was 4 nM. Serum MSP did not increase over a 24 hour period in response to intravenous LPS, indicating that MSP is not an acute phase protein. These findings are consistent with the hypothesis that regulation of MSP activity is by conversion of pro-MSP to MSP, rather than by rapid changes in rates of synthesis.

MSP induces mouse resident peritoneal macrophages to become responsive to the chemoattractant C5a and to ingest C3bi-coated erythrocytes. We have now shown that MSP action is not limited to complement-induced responses, since it also induced responsiveness to the non-complement chemoattractant, casein. In addition to stimulating responsiveness to attractants, MSP functioned alone as a chemoattractant for resident peritoneal macrophages. A critical difference

between MSP and C5a is that resident macrophages did not migrate to C5a without an additional stimulus such as MSP in the cell suspension, whereas macrophages suspended in medium alone migrated to MSP in the attractant well. Thus, in contrast to C5a, MSP appears capable of a dual role, both activator and attractant. MSP had no effect on responsiveness of mouse peritoneal exudate macrophages to C5a; nor could it attract exudate macrophages or human blood monocytes. Absorption studies showed that resident macrophages have a receptor for MSP, but exudate macrophages do not. In view of these findings, it appears that the biological role of MSP is not as a recruiter of blood monocytes to sites of inflammation, but as an activator of mature macrophages. The MSP-induced activated state for responsiveness to C5a or C3bi was transient, and decayed at a first order rate with a half-time of about 1 hour. This is a new example of the transience of activation induced in macrophages by pro-inflammatory stimuli.

In an exploration of other possible cellular actions of MSP, we studied the effect of MSP on cytokine or LPS-induced nitric oxide (NO) synthesis in mouse macrophages. MSP strongly inhibited LPS-induced NO synthesis in a dose dependent manner. Moreover, NO synthesis induced by IFN- $\gamma$  plus IL-2 or TNF- $\alpha$ , which may use different pathways for inducing NO release, was also blocked by MSP. Northern blot analysis showed that MSP suppressed the expression of NO synthase mRNA in both dose and time-dependent fashions. The inhibition is not non-specific, because the expression of monocyte chemoattractant protein-1 mRNA induced by LPS was not affected.

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

PROJECT NUMBER

Z01 CB 08577-08 LIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Restriction fragment length polymorphisms in normal and neoplastic tissues

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

CO-PI: B. Zbar and M. Lerman

Other: F. Latif Visiting Scientist  
 M. Yao Visiting Fellow  
 I. Kuzmin Visiting Fellow

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, MD 21702

TOTAL STAFF YEARS:

7.0

PROFESSIONAL:

5.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

(a) Human       (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 goal of the research effort is to define genes involved in the pathogenesis of urologic malignancies. Our major accomplishment this year is the isolation of the von Hippel-Lindau disease (VHL) tumor suppressor gene. The newly isolated gene appears to play a major role in the pathogenesis of sporadic renal cell carcinoma.

## Major Findings:

A gene discovered by positional cloning has been identified as the von Hippel-Lindau disease (VHL) tumor suppressor gene. A restriction fragment encompassing the gene showed rearrangements in 28 of 221 VHL kindreds. Mapping studies revealed that 18 of these rearrangements were due to deletions in the candidate gene, including 3 large nonoverlapping deletions. Four intragenic deletions and one nonsense mutation were also detected in sporadic renal cell carcinomas. The VHL gene is evolutionarily conserved and encodes two widely expressed transcripts of about 6 and 6.5 kb. The partial sequence of the inferred gene product shows no homology to other proteins, except for an acidic repeat domain found in the procylic surface membrane glycoprotein of Trypanosoma brucei.

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Yao M, Latif F, Orcutt ML, Kuzmin I, Stackhouse T, Zhou F-W, Tory K, Duh F-M, Richards F, Maher E, LaForgia S, Huebner K, Le Paslier D, Linehan M, Lerman M, Zbar B. von Hippel-Lindau disease: identification of deletion mutations by pulsed field gel electrophoresis. *Hum Genet*, in press.

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

PROJECT NUMBER

Z01 CB 08578-04 LIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Preparation of a high resolution genetic map of human chromosome 3

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

CO-PI: B. Zbar and M. Lerman

Other: K Tory PRI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, MD 21702

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

A genetic map has been constructed for the short arm of chromosome 3. The length of the sex-averaged 3p map is 114 cM. The length of the sex averaged 3q map is 123 cM. One hundred and twenty two loci were placed on the short arm of chromosome 3. Forty loci were placed on the long arm of chromosome 3.

## Major Findings:

The three year project for the building of a high resolution genetic linkage map of human chromosome 3 has been completed. During the third year our effort was focused on the development and mapping of new highly polymorphic microsatellite probes instead of the previously studied RFLP markers. Altogether 40 new microsatellite and 150 RFLP markers were placed on the chromosome 3 map reaching an 80 probe/Morgan overall density.

## Publications:

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## LABORATORY OF MATHEMATICAL BIOLOGY

### SUMMARY

October 1, 1992 through September 30, 1993

Research in the Laboratory of Mathematical Biology (LMMB) covers a broad range of theoretical and experimental studies of biological systems. These studies include molecular modelling, theoretical molecular calculations, molecular glycobiology, membrane structure and function, and physiological modeling studies. Basic understanding of these biological systems provides models for aspects of malignant and other disease processes, and is enhanced 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 utilized by researchers in the biomedical community at large. Many of the theoretical studies are possible only through use of the supercomputing facilities at the Frederick Biomedical Supercomputing Center, FCRDC, which was created and sponsored through the initiative of the LMMB.

#### Office of the Chief

Sequence Analyses in Viral, Cellular and Molecular Biology. In the Office of the Chief, computerized analyses are used extensively with data from 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 principles. Detailed studies use structural computations and the effect of point mutations and phylogenetic comparisons. Searches for general trends involve comparisons of structures of related genes.

RNA secondary structures were predicted in correlation with sites involved in internal translational initiation in picornaviruses, infectious bronchitis virus, and pestivirus genomes. Recently this phenomenon was extended to the p53 proto-oncogene. Ribosomal jumping, which occurs in bacteriophage systems thus far seems also to involve unusual mRNA structures. RNA secondary structures have been found that appear to be involved in 5-fluorouracil resistance by thymidylate synthetase. Methods to assess the significance of predictions have used Monte Carlo simulations, evolutionary comparisons and biochemical data (Le, Sonenberg and Chu).

RNA 3-dimensional structures will be necessary for full appreciation of the range of functions of RNA. A program has been developed that uses predicted secondary structures to make plausible 3-D models for graphical display. This is important because there is much less structural information available for RNA than for proteins (Konings and Martinez).

Protein 3-D structure databases are growing rapidly, and tools for efficient quantitative comparison and classification are necessary. Based on principles from computer vision and robotics a method has been developed to make comparisons objective and automatic. Early success at pairwise structure matching allows rapid database comparisons. Further extensions are under way to carry out small molecule docking for drug discovery. A new way to represent molecular surfaces in a sparse and accurate way was developed, and the increased efficiency permits application to larger problems of protein-protein docking (Nussinov, Lin, Tsai, Wolfson, Fischer and Norel).

New analytical tools for studies of protein and nucleic acid sequences have been developed and implemented. 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 Frederick Biomedical Supercomputing Center (FBSC). General patterns are 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, Nussinov, Maizel and Owens).

Information Theory in Molecular Biology. Information theory, invented in the 1940's by Claude Shannon to describe the transmission of information across communication channels, is being used to understand molecular sequence patterns in genetic control systems (Schneider and Hengen). The first results showed that most binding sites contain only the amount of information required for them to be detected in the genome. Unlike several other prokaryotic recognition sites, the sequences at phage T7 promoters have twice the required information. Genetic experiments are carried out to determine the source of this and other anomalies and to determine the structure of the promoters.

A graphical technique, called 'sequence logos' was invented to aid in visualizing the patterns at binding sites. The technique is superior to the use of consensus sequences. The sequence logos are now being used to study the fine structure of binding sites. The fundamental processes of transcriptional control, translation, DNA replication and partition of DNA to daughter cells are actively being studied by using these techniques. Experimental work is also in progress on each of these to test the validity of the theory. The concept of a channel capacity in communication was translated into molecular biological terms. A major result is that we can now explain, on a theoretical basis, why a wide variety of molecules are able to do highly precise things. For example, structures of the restriction enzymes do not directly explain why EcoRI is able to select preferentially only GAATTC from all hexamer sequences. The theory explains this as a coding similar to the error correcting codes used in telecommunications. These approaches set bounds which should aid in the design of molecular devices..

Molecular Biology of Glycosyltransferases. Studies on the structure and function of glycosyltransferases and their interactions with proteins and oligosaccharide ligands are studied. These investigations are directed to probe the interactions of complex carbohydrate structures of glycoconjugates with proteins and how they influence cellular recognition processes.

In order to delineate the functional domains of Golgi glycosyltransferases having inverted membrane topology, the cDNA constructs were expressed, either in mammalian cells or in *E.coli*. The roles of the cytoplasmic and transmembrane domains were examined by transient expression of a series of mutants and chimeric DNA of  $\beta$ -1,4-galactosyl-transferase ( $\beta$ -1,4-GT) in COS-7 cells (Masibay and Qasba). With the help of enzyme activity measurements and localization of the protein in the cell by subcellular fractionation or indirect immunofluorescence microscopy, it was shown that the stable expression and Golgi localization of these enzymes depends on the intactness of the transmembrane domain. By exchanging the topological domains of glycosyltransferases we have identified the membrane anchoring sequences of  $\alpha$ 1-3-galactosyltransferases, and of  $\alpha$ 2-6-sialyltransferase, and have also shown that they are essential for the synthesis and stability of these enzymes in a mammalian system. Analyses of the hydrophobic index of the transmembrane region of Golgi glycosyltransferases versus plasma membrane proteins showed that the Golgi enzymes have overall a shorter hydrophobic length as compared to the plasma membrane proteins. By increasing the hydrophobic length of the transmembrane domain of a Golgi enzyme, by insertion of hydrophobic residues, one can override the Golgi-retention signal and direct the protein toward the plasma membrane.

To analyze the sugar donor/acceptor binding domains of glycotransferases, an enzymatically active  $\beta$ -1,4galactosyltransferase has been produced in *E.coli* (Boeggeman, Balaji and Qasba). Recombinant proteins were localized in inclusion bodies that were solubilized in 5 M guanidine-HCl. Renaturation and regeneration of the enzyme activity, from the solubilized protein was strictly dependent on the presence of an "oxido-shuffling" reagent. Deletion analysis showed that both  $\beta$ -1,4GT and lactose synthetase (LS) activities remain intact even in the absence of the first 129 residues, but the activities are lost when deletions extend to residue 142. Site directed mutagenesis of Cys 134 to either Ala or Ser also resulted in the loss of both  $\beta$ -1,4GT and LS enzyme activities. "Oxido-shuffling" reagents are required for the formation of a disulfide bond involving Cys 134, which is crucial for proper folding of the protein and for the regeneration of the enzyme activity.

To have a precise idea about the bioactive conformer of a given oligosaccharide, it is essential to have the information about all the conformers which are accessible by this particular oligosaccharide. We have applied a molecular dynamics technique to study all the possible conformations of some bi- and triantennary oligosaccharides that are the ligands of glycosyltransferases and of asialoglycoprotein cell surface receptor (ASGP-R) (Balaji, Rao and Qasba). Molecular dynamics simulation of the molecules for one nano-second by considering all the monosaccharides simultaneously has provided a wealth of information about the conformational preferences of these molecules. Contrary to earlier beliefs, our results show that oligosaccharides have a considerable amount

of flexibility. The relative distances and orientations of the galactose residues on the 1,2- and 1,4-branches of the 1,3-arm of triantennary oligosaccharide are invariant during most of the simulation period. Contrary to the 1,3 arm, the 1,6 arm is close to the GlcNAc residues of the core oligosaccharide structure.

Simulation, Analysis and Modelling of Physiological Systems. Development continued on the Simulation, Analysis, And Modeling (SAAM/CONSAM) computer programs (Zech and Greif). The development of a new version of SAAM, which executes on the PC under the DOS operating system and is compatible with MSWindows3.1 has been completed. While rewriting using the DPFI standards, selectable screen control functions specific to the PC were added to CONSAM, and this version was renamed CONWIN. CONWIN, while not yet a true windows program, was modified to take advantage of windows virtual memory management, disk caching of Windows3.1, and the associated SmartDrive utility. Repeated studies have been accommodated, by using the old algorithm in SAAM as a guide. We have rewritten a new algorithm, referred to as EMAS for Extended Multiple Studies Analysis for the automated analysis of the first and second steps of multiple studies data within SAAM.

Demands for additional instruction have occurred because of the improved sensitivity of mass spectrometers and because of the increased availability of labeled precursors with multiple mass shifts which has increased the number of groups undertaking investigations using stable isotope tracer kinetics.

The SAAM project involves collaborative research efforts (Zech), with large numbers of investigators, in the analysis of data in the fields of: lipid and lipoprotein metabolism including reverse cholesterol transport, whole body metabolism and pharmacokinetics of cancer preventive selenium compounds, and the vitamin A dynamics underlying homeostatic mechanisms that function to regulate the general physiological functions of growth and differentiation, reproduction, and vision and their relationships to cancer prevention and in the chemical, pharmacological aspects of cancer chemotherapy and other drugs. This effort also includes clinical duties and responsibilities for radiiodinated tracers for more than 40 lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI.

Reverse cholesterol transport, the only major exit pathway for cholesterol from humans, has long been defined as the uptake of cholesterol from tissues by HDL, the esterification to HDL cholesterol ester, the equilibration of cholesterol ester among the Beta-lipoproteins, and the uptake of this cholesterol via the interaction of HDL with the liver and the secretion of bile acids and biliary cholesterol. Using the modeling approach, two separate collaborative efforts with Drs. Schwartz and Rader, have been combined to make some predictions. This new model predicts that a significant fraction, up to half, of the reverse cholesterol transport path results from the uptake and processing of free cholesterol directly. Taken together, this cholesterol model and the lecithin:cholesterol acyltransferase (LCAT) deficient results predict that the significant fraction of HDL cholesterol ester is associated with the LpAI:AI lipoproteins and free cholesterol transport path results in uptake from the

LpAI fraction of HDL. Improvement and further quantitation of the reverse cholesterol transport pathways involves the design of experiments and building of models to detect further details of cholesterol ester uptake by the hepatocyte.

### Molecular Structure

We are studying the structures and properties of biological macromolecules, including peptides, proteins, DNA and RNA. These studies include the physical chemistry of processes such as folding, binding and conformational changes.

One principal difficulty in achieving the correct folded conformation of a protein is the overwhelmingly large number of possible conformations. Restricting the space to the overall size and shape, for conformation generation, affords a large reduction in the number of feasible folded forms, and hence the computation time. This scheme limits the conformations generated simply by restricting them to be densely packed within a small volume (Jernigan and Covell). It has been possible to enumerate all of the possible folded topologies for several small proteins in several shapes and to evaluate them with simple residue-residue interactions. With a similar approach, we have been studying tertiary folding of RNA (Lustig and Jernigan). The same approach of using regular lattice points to divide and define a conformational space has also proven useful for investigating the binding of small peptides to larger proteins (Covell, Young and Jernigan) and should lead to new methods of drug design.

Molecular modeling has been proceeding in four areas: membrane proteins (Guy, Durell and Raghunathan), small peptides (Jiang and Jernigan), DNA helices (Jernigan, Zhurkin, Jiang and Raghunathan), and DNA-protein interactions (Zhurkin). 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, cecropins, squalamine and ranalexin, these models have improved our understanding of how they lyse cells and form channels. New models have been developed for three groups of potassium channel proteins (voltage-gated, calcium-gated, and inward rectifying potassium channels), using a new structural motif for ion channels in which a  $\beta$  barrel is surrounded by  $\alpha$  helices. A model of channels formed by beta amyloid protein has also been developed. This model may provide insight into the role of beta amyloid protein in Alzheimer's disease. Structural details of DNA double helices exhibit some dependence on the base sequence; these 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. Models of three-stranded and four-stranded DNA helices are being developed, and the function of one of them for recombination is being investigated. Subjects being studied include symmetry and regularity, specific ion stabilization and interstrand interactions.

## Image Processing

Massively parallel computation has been a significant portion of the past year's research effort. We have been researching, testing and upgrading a massively parallel computer architecture. The acquisition and upgrade of a MasPar MP-1 to a MasPar MP-2 has changed the way many computational problems within our laboratory are being thought about. The MasPar MP-1 has been upgraded to a 16,384 processor MP-2 with a 6 Gigaflop peak performance capability. Several algorithms have been developed and adapted to the MasPar including the newly developed genetic algorithm, a very fast version of the suboptimal dynamic programming algorithm for RNA structure prediction, a very fast and sensitive sequence comparison algorithm for determining sequence homologies in proteins and nucleic acids, and a visual docker for docking drugs with a protein substrait (Shapiro, Navetta and Maizel).

The first phase of research into a new class of algorithms, "genetic algorithms", for RNA folding is being completed (Shapiro and Navetta). Because of the highly parallel nature of the algorithm it lends itself well to the MasPar architecture. It computes 16,384 RNA conformations at each generation. In several tests the algorithm converges to optimal or almost optimal conformations. The genetic algorithm uncovered some unknown bugs that existed in a commonly used dynamic programming algorithm by finding structures that had better free energy values than the dynamic programming algorithm was producing.

In addition to the genetic algorithm, a massively parallel sequence comparison algorithm, BLAZE, is running on the MasPar. It is capable of performing amino acid comparisons at the rate of 220,000,000 residues per second with 16,384 processors. This comparison includes affine gap penalty calculations which improves sensitivity. Speed and sensitivity are becoming more important for sequence analysis as the size of the sequence databases grow (Shapiro, Smythers, Gunnell).

We also have ported to the MP-2 the Zuker suboptimal RNA folding code which is capable of folding large RNA sequences up to about 9400 nucleotides in length without any special packing. This has permitted the folding of such sequences as HIV and rhinovirus. This is larger than the CRAY YMP is capable of folding. It has also been demonstrated that the MP-2 can fold such sequences faster than a single processor of the CRAY YMP thus giving a considerable price performance advantage (Shapiro, Chen, Navetta, Kasprzak and Maizel).

Work has also continued on the heterogeneous RNA structure analysis system with improvements in its graphical presentation capabilities, RNA database matching facilities, mutated structure generation and extensions to the MasPar interface. For example, the MasPar with the currently running versions of the genetic algorithm for RNA structure prediction and the fast suboptimal dynamic programming algorithm has been incorporated into this heterogeneous computing environment. This system has been developed for the analysis of RNA secondary and tertiary structure and runs on a SUN workstation. 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 and tertiary



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 and tertiary structure problem domain involving various software/hardware complexes available at the Frederick Cancer Research and Development Center and elsewhere (Shapiro and Kasprzak).

The above system in conjunction with gel shift experiments has been used to help determine the binding site of nucleocapsid protein NCp7 of HIV-1 and the RNA structural components that determine this site. This protein is important for encapsidation of the virus genome, RNA dimerization and primer tRNA annealing in vitro. Results show that NCp7 binds to a unique RNA structure within the Psi region. Also, this structure is necessary for RNA dimerization. It is proposed that NCp7 binds to the RNA via a direct interaction of one zinc binding motif to a stem loop structure in one RNA molecule followed by binding of the other zinc binding motif to a motif in the other RNA molecule (Shapiro and Baldwin). The determination of the RNA structure discussed above was accomplished using several features within the RNA structure analysis system.

The system is also being used to help determine the structure of human cytochrome mRNA which contains internal "inframe" UGA codons of unique dual function: termination of translation or insertion of selenocysteine. Unique RNA structures have been found in the 3' untranslated region of the molecule. The mRNAs of known selenocysteine-containing proteins share the selenocysteine insertion sequence (SECIS) motif and demonstrate a predictable folding into a somewhat unique stem-loop structure located at their 3' untranslated region. We searched for the SECIS structural motif in CYP2B7 mRNA (2907 nucleotides). A loose structural pattern was specified based upon the published structural motif and this was run against a database of 500 optimal and suboptimal RNA secondary structures. We located an area in the 3' untranslated region that had similar characteristics to that of the published selenocysteine structures (Shapiro, Czerwinski and Kasprzak).

A new algorithm for discovering motifs in protein sequences had been developed and is undergoing testing. It uses the concept of developing the motifs from a small sample of database sequences and then refining these motifs by running them against sequences in the database. Experimental results of running this algorithm on three protein families are giving good results (Shapiro and Shasha).

Analytic methods are continuing to be developed for the GELLAB-II software system with concentration on increasing: quantitation accuracy and speed, improved X-window graphical user interfaces for easier investigator use. 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 (Lemkin, Wu and Upton). 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. We have been executing a CRADA agreement to commercialize GELLAB-II that will result in its much wider availability using an inexpensive Windows-NT PC platform and provide better user support

than we can supply for large numbers of users. A major thrust of the work this year was in working with our CRADA partner in refining and transferring the GELLAB-II technology to insure a smooth process during the commercialization of GELLAB-II (Lemkin, Wu and Upton). We have been collaborating in developing a protein-disease relational database system with respect to a reference 2D gel for plasma and serum. This system will be able to answer questions of the form which what diseases change with these proteins and vice versa and where are they in the reference 2D gel (Lemkin and Merrill). We have continued our work on remote collaborative multimedia image-conferencing using our Xconf system that allows GELLAB-II as well as a broad range of images from other fields and software to be shared over the national Internet computer network (Lemkin). Active GELLAB-II collaborative work has continued with the groups of: Dr. J. Myrick (CDC/Atlanta); Dr. C. Merrill et.al. (NIMH); Dr. P. Rogan (Penn State Med. Sch.); Dr. T. Krekling (Agr.U.Norway); Dr. R. Leimgrubber et.al. (Monsanto Co.).

#### Membrane Structure and Function

The research goals in the Membrane Structure and Function Section (Blumenthal, Puri, Krumbeigel, Pak, Elson and Dimitrov) are directed towards an understanding of mechanisms of membrane fusion mediated by viral spike glycoproteins. The mode of action of the envelope protein of HIV 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; and 13) development of methods for using reconstituted viral envelopes as vehicles for specific delivery of materials into cells.

The first step of entry of enveloped animal viruses into cells is by fusion of the membrane of the virus with that of the target cell. This fusion process is catalyzed by viral envelope proteins. We have developed biophysical techniques to study the initial steps of viral envelope protein mediated membrane fusion. We label intact virus or cells with fluorescent dyes and observe the redistribution of those dyes during the fusion

process. We study initial steps of HIV envelope protein-mediated membrane fusion, by continuous monitoring of fluorescent dyes during fusion using fluorescence spectroscopy and low light, image enhanced videomicroscopy. In particular we use HIV envelope protein expressed in cells by means of recombinant vaccinia virus and target membranes of defined composition with and without CD4 receptors. In this way we monitor fusion between cells or syncytium formation. The combination of studies employing HIV-expressing effector cells and defined target membranes facilitates the testing of hypotheses regarding the role of different factors in adhesion and fusion. Transmission of retrovirus between cells is thought to be associated with cell membrane fusion. In this way the virus is not exposed to the extracellular space and thereby hidden from the immune response. Thus, membrane fusion is a key element in the pathology of HIV, and an understanding of the mechanism of viral fusion might lead to the development of anti-viral therapeutic agents.

### Membrane Biology

Work in the Membrane Biology Section was distributed into seven main projects, most of them in collaboration with scientists at NIH as well as abroad. These studies link our expertise in immunogold labeling electron microscopy and in a unique system of methods developed in our laboratory over the past decade (fracture-label, label-fracture, fracture-flip, simulcast) with the research needs of scientific groups that lack expertise in these areas. Our methods address questions of topobiology that are becoming more and more important now that the basic molecular biology questions are being routinely solved and where questions on the regulatory and/or modulating cellular biological aspects must be answered. The main areas of research are: (A) immunogold cytochemical localization of oncogenes and oncogene-related proteins (Rulong); (B) immuno-cytochemical study of retroviral infection (Risco); (C) effects of the interaction of bacterial endotoxins with the plasma membrane of macrophages and pneumocytes (Risco); (D) nanoanatomy and topochemistry of the cell surfaces of protozoan parasites (Pimenta); (E) ultrastructural aspects of 67 kD laminin receptor and its precursor processing in metastatic potent cells (Romanov); (F) freeze-fracture immunocytochemical study of the expression of native and recombinant GABA<sub>A</sub> receptors (Caruncho); and (G) ultrastructure and response to darkness of goldfish meninges (Caruncho).

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

PROJECT NUMBER

201 CB 08300-21 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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 LMMB, NCI  
 Detail from OD, NHLBI

OTHER PROFESSIONAL PERSONNEL:

Peter C. Greif Computer Programmer Analyst LMMB, NCI

COOPERATING UNITS (IF ANY)

Dr. Ray Boston and Charles Ramberg, Univ. PA, New Bolton, PA; Dr. Charles Schwartz, Med. College of VA, Richmond, VA; Dr. Waldo R. Fisher, Univ. of FL, Gainesville, FL; Juerqan Schafer, Klinikum Der Philipps-Universitat Marburg, Marburg Germany; (Contd)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.9

PROFESSIONAL:

1.9

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

A & B

SUMMARY OF WORK (USE STANDARD UNREDUCED TYPE. DO NOT EXCEED THE SPACE PROVIDED.)

DOS-Windows 3.1 environment enhances DOS by allowing more than one application to appear to run simultaneously. We rewrote the I/O portion of SAAM and CONSAM using the DPMI standards incorporating selectable screen control functions specific to the PC. This version of CONSAM was renamed CONWIN, and has screen control functions for background and foreground color selection, number of lines on the screen, scrolling, number of lines in the scroll, number of command lines displayed, command line recall, and command line editing.

Identifying three stages which investigators follow in multiple studies analysis as, first, attempts to find a structurally consistent kinetic model for each study; second, estimated individual parameter values are combined to produce a single population estimate; and third, individual data sets are compared to the population model with a view to locating potential subpopulations and outliers. We have written new SAAM31 algorithms, referred to as EMAS for Extended Multiple Studies Analysis for the automated analysis of the first and second steps.

Recently we have discovered that the metabolism of LpAI:AIII is increased in subjects with complete or partial lecithin:cholesterol acyltransferase (LCAT) deficiency. The mean plasma residence time of plasma apoAII was reduced 50%, and the apoAII production rate is not significantly different from normal controls. Hence, LCAT and the LpAI:AIII lipoprotein play a central role in reverse cholesterol transport. When combined with a new free cholesterol model that predicts that a significant fraction, up to half, of the reverse cholesterol transport path results from the uptake and processing of free cholesterol directly, the hypothesis that free cholesterol is removed preferentially from the LpAI subpopulation of HDL particles is easily postulated.

**Cooperating Units (Continued):**

Marburg Germany; Drs.. H. Bryan Brewer and Daniel Rader, Molecular Diseases Branch, NHLBI; Richard E. Gregg, Squibb Inst. for Med. Res., Princeton, NJ; Dr. Blossom Patterson, Operations Research Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA; Dr. Kevin C. Lewis, Human Nutrition Laboratory, NCI; Drs.. Ba-Bie Teng and Allen Sniderman, Royal Victoria Hospital, Montreal, Quebec, Canada; Dr. Ahmed Kissebah, Univ. of Wisconsin, Madison, WI; Dr. Andre J. Jackson, FDA, Rockville MD; Dr. Mary Mckenna, Univ. of Maryland Medical School, Baltimore MD; and Drs.. Barbara V. Howard and David C. Robbins, Medlantic Research Foundation, Washington DC.

**PROJECT DESCRIPTION****Project #1:**

The continued 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. SAAM and CONSAM are the group of computer programs which facilitate the building and testing of compartmental models.

**Major Accomplishments:**

1. **SAAM and CONSAM Development.** When SAAM and CONSAM were migrated from the VMS and Unix operating systems to the MSDOS operating system on '386/486 computers we chose to execute in the 386 Protected Mode by using 32bit compilers and a DOS extender to provide an interface between the SAAM application and DOS operating-system services such as I/O and other real-mode function libraries. Extended SAAM/CONSAM for the 386 was written for a flat (unsegmented) address space using up to 5MB of memory avoiding the 64KB-segment limit of the '286. This has the advantage that it could be transparent to the DOS users and would create a thousand or more new users of the program. This technique also resulted in several disadvantages, more than 4MB of extended memory are required, and a math coprocessor is required. This migration made extensive use Virtual Control Program Interface (VCPI) methods and VCPI programs are not compatible with Windows 3.1

DOS-Windows 3.1 environment enhances DOS by allowing more than one application to appear to run simultaneously. Windows 3.1 does not support VCPI standard but uses DOS Protected Mode Interface or DPMI. The migration of SAAM and CONSAM to the DPMI environment is conveniently divided into two parts, moving to the DPMI memory management and I/O functions, and the second step is to build a complete windows graphical interface. The first part of this migration has been accomplished this year.

While rewriting the I/O portion of CONSAM using the DPMI standards, selectable screen control functions specific to the PC were added to CONSAM, and this version was renamed CONWIN. The screen control functions include background

and foreground color selection, number of lines on the screen, scrolling, number of lines in the scroll, number of command lines displayed, command line recall, and command line editing.

After developing and testing a MS-Windows 3.1 compatible DOS version of SAAM and CONSAM, and the advent of the 25 and 33 MHz '486SX computers, two additional disadvantages came more clearly into focus. Now that the program was compatible with DPMS it was possible to further adjust the memory management scheme to include virtual memory management (VMM). CONWIN, while not yet a true windows program but a DOS program launched from windows, was modified to take advantage of windows virtual memory management and diskcaching of Windows 3.1 and the associated SmartDrive utility. This improvement makes CONWIN available to '386 and '486 users with less than 4MB of memory as long as it is launched from the Windows 3.1 File Manager.

The Intel '486SX and the IBM '486SCL microprocessors have become a frequent choice within the scientific community; however, both lack the implementation of a math co-processor. Because of the popularity in portable computers and the increased computing power of this class of PC, frequency of request for copies of SAAM to execute on this type of PC has increased. SAAM and CONWIN were further modified to use an emulator for the math co-processor when needed. This resulted in a tradeoff between speed of execution and the number of potential users.

Effective quantitation of kinetic data resulting from tracer studies of metabolic systems ideally calls for an integrated array of computer based modeling tools in juxtaposition with enlightened study design. SAAM was originally assembled beginning almost 30 years ago by Mones Berman specifically to harness appropriate numerico-statistical techniques for the direct and efficient interpretation of kinetic data in terms of compartmental models. Early computer systems supporting the SAAM software were strictly the province of major computing centers; but now, for little money an investigator has access to the SAAM software on his or her laboratory bench. This has implications from the perspective of SAAM's development and extension. One direction is the development and implementation of software associated with data analysis pertaining to multiple studies or repeated experiments of the same design in several subjects.

The objective of repeated experiments is to characterize a population in the epidemiological sense while isolating studies or experimental results which for some reason produce aberrant responses. The analysis of multiple studies advances in three stages: first, the investigator attempts to find a kinetic model structurally consistent with each study; second, the estimated parameter values are combined (averaged) to produce a single population estimate; and thirdly, each individual data set is compared to the population model with a view to locating potential subpopulations. In compartmental modeling several problems arise in the computational analysis including differing precise estimates of model parameters among data sets, studies are not necessarily homogeneous, and parameters of the model are not independent of one another. This means that simply averaging the individual parameters estimates can yield

spurious results irrespective of what weighing mechanism is used. To obtain unbiased results for the population other methods are developed.

In addition to having made several major adjustments to the memory management, a major reduction in the hardware requirements for SAAM31 and CONWIN execution, and significant corrections in the EMSA algorithms we and the community of users have detected more than 2 dozen errors in the software which have been corrected. The above changes together represent the 31st major revision of SAAM and the corresponding interactive environment CONWIN.

**2. SAAM Workshops & Distribution.** In the past year we have been involved in several workshops. In conjunction with the American Institute of Nutrition we organized a teaching session on the development of compartmental models for beginners preceding the 1993 FASEB meeting. In conjunction with the American Physiologic Society we also participated in a computer demonstration area where several hundred researchers came to ask questions about SAAM and CONWIN at the 1993 FASEB meeting. We organized a SAAM workshop in Marburg Germany for European investigators.

Many 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. This involves combining and confronting theorist and experimentalist with topics which can profit from the application of computer simulation and computation. This further serves to obtain the best experimental data for analysis and inclusion in data bases, such as the lipoprotein, retinoid, and selenium data bases. To this end we have extended the plan to continue to distribute these programs as necessary in the scientific community. SAAM and CONWIN have been made available anonymously over the INTERNET network or by modem.

#### Project #2:

Application of SAAM and CONSAM to the Simulation and Analysis of Bio-kinetic Data. Partially with the aim of assuring that the Bio-Kinetic data collected will be applicable to compartmental analysis, this effort has included 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, as the NIH authorized user, for 45 lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI.

#### Major Findings:

1. In collaboration with Charles C. Schwartz: Reverse cholesterol transport, the only major exit pathway for cholesterol from humans, has been defined for several decades as the uptake of cholesterol from tissues by HDL, the esterification to HDL cholesterol ester by LCAT, the equilibration of

cholesterol ester among the Beta-lipoproteins via CETP, and the uptake of this cholesterol via the interaction of HDL with the liver (proposed receptor) as well as uptake of LDL by the hepatic LDL receptor and the formation and secretion of bile acids and biliary cholesterol. Because of its importance in atherosclerosis, gallstone formation, and the metabolism of lipid soluble carcinogens, the quantitation and modulation of the Reverse Cholesterol Transport pathway is of major interest. One simple question under investigation is; what contribution does free (unesterified) cholesterol make to Reverse Cholesterol Transport pathways?

Based on insights gained from previous experiments studies were designed and carried out in an experiment to detect: (a) transport of free cholesterol between the  $\beta$  lipoprotein fraction, liver, and other tissues; (b) unidirectional (net) free cholesterol transport to or from HDL over and above the exchange between HDL, liver, other tissues, and lipoprotein fractions; (c) accurate estimate of tissue pool size. This was achieved by administration of multiple isotopes, rapid separation of HDL from  $\beta$  lipoproteins (less than 5 minutes after collection of blood samples), and sampling lipoprotein, red cell, and biliary free cholesterol; lipoprotein cholesterol ester, and bile acids.

The only major exit of cholesterol from the human body is through the hepatic bile acid, biliary cholesterol path. This path was determined to be up to 6  $\mu\text{mol}/\text{min}$  in bile fistula subjects. There is net transport of cholesterol from the HDL plasma to the hepatic free cholesterol pool of almost 8  $\mu\text{mol}/\text{min}$  in addition to a transport of 13  $\mu\text{mol}/\text{min}$ . A rudimentary cholesterol ester model suggest that 2 to 3  $\mu\text{mol}/\text{min}$  of cholesterol ester moves from the plasma to the hepatic precursor pool. Taken together this model would predict that a significant fraction, up to half, of the reverse cholesterol transport path results from the uptake and processing of free cholesterol directly. Improvement and further quantitation of the reverse cholesterol transport pathways involves the design of experiments and building of models to detect the details of cholesterol ester uptake by the hepatocyte.

**2. In collaboration with Dr. Daniel Rader and others in the molecular disease branch of NHLBI:** In the process of transporting lipids, plasma lipoproteins are subjected to a series of enzymatic reactions and physical-chemical processes that have been examined in detail in vitro. The physiology of the plasma lipid transport system may also be examined in vivo utilizing tracers. Measurements obtained in kinetic studies translate the knowledge of lipoprotein metabolism at the molecular level into understanding of the normal and by comparison the altered physiology occurring in specific diseases.

In the past 10 years since this laboratory predicted that a rapidly metabolized HDL fraction containing apoAI and not apoAII represented a significant fraction of HDL metabolism, the metabolism of LpAI (apoAI only lipoprotein) has been investigated by many laboratories. Investigation of the HDL fraction containing both apoAI and apoAII, LpAI:AII, has been neglected because there



was no disorder in which this lipoprotein was changed. Recently we have discovered that the metabolism of LpAI:AIII is increased in subjects with complete or partial lecithin:cholesterol acyltransferase (LCAT) deficiency. As HDL accepts excess unesterified cholesterol from peripheral cells the HDL free cholesterol is esterified by LCAT. Hence, LCAT and the LpAI:AIII lipoprotein are believed to play a central role in reverse cholesterol transport. In order to gain further insight into reverse cholesterol transport, and the metabolic bases of the hypoalphalipoproteinemia in LCAT deficiencies the kinetics of plasma apoAI and apoAII as well as LpAI and LpAI:AIII HDL lipoproteins was investigated. The concentrations of LpAI:AIII HDL lipoproteins was reduced. The mean plasma residence time of plasma apoAII was reduced 50% to  $1.66 \pm .24$  days. Because the apoAII production rate of  $2.55 \pm .87$  mg/kg-d is not significantly different from normal controls, the selective increase in metabolism apoAII and probably the selective metabolism of the LpAI:AIII HDL fraction is responsible for the lack of corresponding coronary artery disease in this group of subjects.

3. In collaboration with Dr. Kevin C. Lewis, of the Laboratory of Molecular and Nutritional Regulation: Lipid soluble retinoids are carried and metabolized in the lipoprotein system and play a major role in the promotion of growth and differentiation in epithelial tissues, hence, the interest from both the carcinogenic prevention and lipid metabolism views. The long term objective is to understand the role of other retinoids to substitute for vitamin A in the deficient abetalipoproteinemic and provide optimal cancer prevention in the adult. While most studies have focused on the pharmacokinetics of 4-HPR we have begun to investigate the kinetics of native vitamin A metabolism under the influence of 4-HPR. In rats, a decrease was observed in the area under the plasma decay curve of radiolabeled native vitamin A following treatment with 4-HPR. This finding suggest that the tissue distribution, metabolism, and storage of retinol is completely rearranged by administration of this medication. A complete kinetic system is being designed to examine and compare tissue levels of retinol in control and 4-HPR treated animals as well as the comparison of the excretion of metabolic products of retinol in urine and feces. The long range goal of this collaboration is the study of retinol metabolism in both normal subjects, retinol deficient patients such as abetalipoproteinemia, and subjects with tumors.

4. In collaboration with Dr. Blossom Patterson, Operations Research Branch, NCI; Drs. Phil Taylor and Christine Swanson, 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 pharmacokinetics 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 is much more complex than was originally thought when the study was designed. This has led to the application of kinetic modeling to understand the details. The process of building a kinetic model for the metabolism of selenite and selenomethionine in humans is continued with the goal that when the models for the organic and inorganic forms have been completed they can be combined with the help of the computer to estimate the kinetics of a physiologic dose of selenium made up of both organic and inorganic forms.

The previously developed kinetic model for selenite metabolism was applied to a portion of the main study where the 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 a steady state. A split unit design was chosen and 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.

There appears to be a difference in the two most rapid plasma components as a function of the fasting status. In a crossover study in which each subject also serves as a control, there is always the possibility of carryover, i.e., 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. A model for the analysis of the kinetics organic selenium, selenomethionine has been completed using the first six 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. Based on this model, it is expected that the carryover will be even larger than in the case of the inorganic selenium but, the effects of pre-study body burden less. When the details of both the inorganic and organic selenium compounds have been determined, further analysis of this data set will center around the comparison of inorganic and organic forms. As the physiologic intake of selenium is a mix of both organic and inorganic forms, and both can not be examined simultaneously, this can only be accomplished with computer simulation.

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

PROJECT NUMBER

Z01 CB 08303-21 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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 & Function Sect., LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Anu Puri, Ph.D.	Visiting Associate	LMMB, NCI
Charles Pak, Ph.D.	IRTA Fellow	LMMB, NCI
Mathias Krumbiegel, Ph.D.	Visiting Fellow	LMMB, NCI
Dimitter S. Dimitrov, Ph.D.	Visiting Scientist	LMMB, NCI
Hannah Elson, Ph.D.	Expert	LMMB, NCI

COOPERATING UNITS (IF ANY)

Dr. Hana Golding, CBER; Dr. Joel Lowy, AFFRI; Dr. Michel Ollivon and Marie-Therese Paternostre, CNRS, France; Drs. Abraham Loyter and Lev Bergelson, Hebrew University, Israel; Dr. Joshua Zimmerberg, NICHD; Drs. Yi-der Chen, F. Boov and (continued)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Structure and Function Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

5.0

PROFESSIONAL:

5.0

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.)

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 envelope glycoproteins of Human Immunodeficiency Virus, the G protein of Vesicular Stomatitis Virus, the HN and F proteins of paramyxovirus, and the Hemagglutinin of influenza virus. The fusion process involves a range of steps before the final merging of membranes occurs. Our studies deal with a number of key questions concerning the fusion process such as: How does triggering the event by a pH or temperature change, or receptor binding affect conformation of the viral envelope protein?; How do the viral proteins mediate adhesion at the site at which fusion is to occur and movement of membranes into apposition?; Can we identify intermediate fusion steps or structures?; What sorts of molecular rearrangements occur before, during and after the fusion event? Those questions are approached by developing kinetic assays for fusion of fluorescently-labeled virus with a variety of target membranes using spectrofluorometric and video microscopic techniques. We continuously monitor fluorescence changes before and during fusion. We also examine fusion activity of viral proteins expressed on surfaces of cells. The monitoring is done on single cells as well as on cell populations using different criteria for fusion (cytoplasmic continuity and lipid mixing). Using a variety of biophysical, biochemical and molecular biological techniques we analyze steps in viral envelope protein-mediated fusion which include conformational changes, activation, fusion pore formation, the actual merging of the membranes and the wide opening which allows delivery of the nucleocapsid into the cell. The parameters and "design principles" derived from studies with viral envelope proteins provide a conceptual basis for constructing synthetic plasma membrane fusion proteins which may be used as components of targeted systems negotiating entry of therapeutic agents into cells.

## Cooperating Units (continued):

Yossef Raviv, NIDDK; Drs. E. Berger, B. Moss, C. Broder, R. Willey, M. Martin, NIAID; Dr. S.J.Morris, UMKC, Kansas City; Dr. D.P. Sarkar, Univ. of New Delhi, India; Dr. Rauf Giurguis, Cancer Diagnostics Inc., Fairfax, VA.

## PROJECT DESCRIPTION

Major Findings:

1. **Redistribution of viral lipid, protein and RNA upon influenza virus fusion**

We examined by video microscopy the redistribution of influenza hemagglutinin (HA), viral lipid, and RNA upon incubation of influenza-erythrocyte complexes at low pH and different temperatures. Lipid was monitored using the fluorescent dye, R18, influenza HA was observed by light and electron microscopy following immunochemical staining or following labeling with FITC, and viral RNA was followed by staining with acridine orange. Redistribution of lipid, HA and RNA as a result of low pH-induced fusion occurred above 20°C with different rates. However, at temperatures below 15°C, low pH incubation for >8 min resulted in lipid redistribution, whereas HA remained with the virus. Release of viral RNA occurred under conditions where the viral envelope was still clustered. These studies are consistent with differential dispersion of viral components into the erythrocyte and existence of relatively long-lived barriers to diffusion subsequent to the formation of fusion pores.

2. **Formation of multiple small pores in influenza virus fusion**

We have measured membrane mixing, cytoplasmic mixing and cell capacitance changes resulting from low pH-induced fusion of influenza HA- expressing cells with erythrocytes. Reversible fusion pore formation with steps in conductance was detected prior to either membrane or cytoplasmic redistribution. Membrane lipid redistribution preceded redistribution of molecules of low molecular weight ( $M_r > 340$ ). The observation that small molecules fail to move between the cells when the total conductance of a single pore is large enough to account for permeability of small molecules indicates that the fusion event is associated with the opening of multiple small pores.

3. **The role of the fusion peptide sequence in initial stages of influenza hemagglutinin-induced cell fusion**

The fusion activity of influenza hemagglutinin (HA) and of HA proteins altered in the amino-terminus of HA2 (fusion peptide) by site-directed mutagenesis (Gething et al, J Cell Biol 102, 11-23,1986) was analyzed following expression

in CV-1 cells using SV40-HA recombinant virus vectors. Fusion was monitored by the redistribution of lipid and cytoplasmic dyes between fluorescently labeled erythrocytes and HA-expressing CV-1 cells using spectrofluorometry and fluorescence microscopy. The kinetics of lipid redistribution after lowering the pH showed the same pattern for wild type HA and non-lethal mutants, although there were shifts in the pH threshold. The time for commitment to the fusogenic state and the temperature dependence of the processes leading to HA-mediated fusion were also the same for wild type and non-lethal mutants. However, striking differences were observed between wild type HA and the non-lethal mutants in their ability to induce pH-dependent redistribution from erythrocytes to HA-expressing cells of large molecular weight ( $M_r > 10,000$ ) fluorescently labeled dextran molecules. The data indicate that the kinetic processes which are measurable in the time range of seconds are insensitive to the structure of the fusion peptide. Surprisingly, however, the fusion peptide plays an important role in later processes related to pore widening which eventually results in delivery of the nucleocapsid into the cell.

#### 4. Rate-limiting step for hemagglutinin mediated fusion differs for PR/8 and X31 influenza strains

The fusion kinetics with erythrocyte ghosts of two influenza A strains, A/Aichi/2/68 (X31) and A/PR/8/34 (PR/8), were compared and correlated with the kinetics of the HA conformational change. Although the pH dependence of fusion at 37°C, monitored by the dequenching of octadecylrhodamine (R18), was quite similar between the two strains, there was a marked difference in temperature dependence at optimal pH. At 25 and 37°C fusion was initiated within 1 s after exposure of the virus-erythrocyte complexes to low pH and completed with 5 minutes. The rates and extents of fusion were virtually the same for the two virus strain at those temperatures. However, at 4°C fusion of X:31 was only observed after a lag time of 5-10 min and completed with 60 min, whereas PR/8 displayed no fusion at that temperature. Moreover, in contrast to PR/8, X31 could be committed to fuse at neutral pH and 37°C by a pre-incubation at low pH and 4°C. The temperature dependence of fusion of the two influenza virus strains correlated quite well with that of low pH-induced binding to liposomes, which is a measure of a conformational change which renders HA more "hydrophobic." However, exposure of the fusion peptide and dissociation of the globular head regions, as measured by two acid conformation-specific antibodies, were rapid for both PR/8 and X31 strains even at 4°C. This study reveals an intermediate step in the overall cascade of events leading to influenza HA-mediated membrane fusion which involves exposure of the fusion peptide at low temperature without interaction of HA with the target membrane.

#### 5. Thermodynamic studies of influenza HA

The conformational stability of HA from influenza strain X:31 was investigated by differential scanning calorimetry to characterize thermodynamically the

structural change accompanying the unfolding process. DSC profiles of purified HA rosettes reveal a single endotherm at a transition temperature of 66.5°C with an enthalpy change of  $\Delta H_{\text{cal}} = 980$  kcal/mol. Deconvolution of the HA endotherm indicates that the protein unfolds in a cooperative manner which may be described by three two-state transitions. Evidence for three thermodynamic domains is consistent with the calculated cooperative ratio of  $\Delta H_{\text{cal}}/H_{\text{vH}}=3$ . The temperature stability of the protein is significantly reduced at pH 4.9 and the enthalpy of melting is three times smaller than that of the native protein. Moreover, the low pH form of hemagglutinin preserves its native far UV CD spectrum, while its near UV CD spectrum is essentially reduced. These data are consistent with the notion that the low pH induces a transition of a substantial part of HA into "molten globule" state, and that this transition may trigger the release of the fusion peptide followed by its penetration into the target membrane.

#### 6. Photosensitized labeling of viral envelope proteins before and after fusion

We have applied a new methodology, called photosensitized labeling, to study initial steps of viral envelope protein mediated fusion. Dr. Raviv has pioneered this approach to the study of recognition phenomena in membrane and cell biology. By this methodology, lipophilic aryl azides are photoactivated *in situ* by energy transfer from a variety of chromophores, using visible light. We could show that insertion of the fusion peptide of influenza hemagglutinin (X:31 strain) into biological target membranes at low pH is rapid and occurs well before the actual merging of membranes. We are currently applying this technique to examine insertion of the fusion peptide of gp41 into target membranes before and after HIV-1 envelope glycoprotein-mediated fusion of CD4<sup>+</sup> cells.

#### 7. Reconstitution of paramyxo-viral envelopes

Viral envelopes containing the fusion protein (F) and hemagglutinin-neuraminidase (HN) (F,HN-virosomes), or F only (F-virosomes) were constructed by dissolution of the virus in detergent followed by purification of the envelope proteins, and removal of the detergent. Using an assay based on the relief of self quenching of a lipid probe incorporated in the Sendai envelopes we demonstrate the fusion of both F,HN-virosomes and F-virosomes with cultured HepG2 cells containing the asialoglycoprotein receptor, which binds to a terminal galactose moiety of F. By desialylating the HepG2 cells the entry mediated by HN-terminal sialic acid containing receptor interactions was bypassed. We show that both F-virosomes and F,HN-virosomes fuse with desialylated HepG2 cells, although the rate was 2-3 fold faster if HN was included in the viral envelope. We also observed enhancement of fusion rates when both F and HN envelope proteins were attached to their specific receptors.

Description of AIDS Research

## 8. Role of CD4 structure in HIV-1-Env glycoprotein-mediated fusion

Several domains of CD4 have been suggested to play a critical role in events following its binding to the HIV-1 envelope glycoprotein (gp120-gp41). Cells, expressing a chimeric molecule consisting of the first 177 residues of human CD4 attached to residues from the hinge, transmembrane and cytoplasmic domains of the human CD8, did not fuse with HIV-1 infected cells (J Virol 65:4893-4901). We found that the hybrid CD4.CD8 molecule expressed in human cells did render them susceptible to fusion with cells expressing HIV-1 IIIB or HIV-1 RF envelope glycoproteins encoded by vaccinia recombinants. After prolonged periods of cell co-culture (24 h) the number of syncytia in both cases did not differ more than 2-3 fold. The lag time of membrane fusion mediated by the hybrid CD4.CD8 molecule, however, was about 10-fold longer than that for the wild type CD4 molecule as measured by a fluorescence dye redistribution assay. The initial rates of fusion mediated by the CD4.CD8 molecule were about 5-fold lower than those for the wild type CD4. The rate of binding and affinity of soluble gp120 to membrane-associated CD4.CD8 were the same as those of CD4. Both molecules were laterally mobile as determined by capping experiments. Co-expression of the CD4.CD8 chimera with wild type CD4 did not lead to interference in fusion, but rather resulted in an additive effect. We conclude that the proximal membrane domains of CD4 play an important role in determining the rate of post-binding events leading to membrane fusion, probably by affecting the rate of conformational changes of the CD4-gp120-gp41 complex.

## 9. Kinetics of HIV-1-Env glycoprotein-mediated fusion of single cells

The kinetics of fusion of cells expressing gp120-gp41 with CD4<sup>+</sup> target cells was continuously monitored by image enhanced Nomarski differential interference contrast optics. The analysis of the video tape recordings showed several characteristic features of the gp120-gp41-mediated cell fusion: (i) cells made contact relatively rapidly (within minutes), in many cases by using microspikes to "touch" and adhere to adjoining cells, (ii) the adhered cells fused after a relatively long "waiting" period, which varied from 15 min to hours, (iii) the morphological changes after membrane fusion, which led to disappearance of the interface separating the two cells, were rapid (within a minute), and (iv) the process of syncytia formation involved subsequent fusion with other cells and not simultaneous fusion of many cells. The kinetic dissection of steps allows us to determine at what stage of the process antiviral intervention is optimal.

## 10. Photodynamic inactivation of viral fusion

Recently, a new membrane dye, PKH26, which is very stable and allows monitoring of labeled cells in vivo for prolonged periods of time, was developed. We



found that photoactivation of this dye by illumination with green light inhibits gp120-gp41-mediated cell fusion at an early stage. The inhibitory effect was localized in space and could be precisely controlled in time. Similar effects were observed with the water soluble dye BCECF. Using the photodynamic inactivation technique we have developed a new approach to measure the kinetics of initial stages of entry of virus (HIV-1 and vaccinia) into cells.

#### **Interactions of CD4 bearing plasma membrane vesicles with gp120-gp41 expressing cells**

We have developed plasma membrane vesicles (PMV) bearing appropriate receptors as a new system to measure fusion activity of cloned viral envelope proteins, and as an agent for inhibition of viral infection (Puri et al, J AIDS, 5:915-920 (1992)). CD4-PMVs were prepared by hypotonic lysis from the cells expressing high amounts of CD4 following infection with recombinant vaccinia. Fusion kinetics between PMV and cells expressing viral envelope glycoprotein was investigated by utilizing a lipid mixing assay based on fluorescence dequenching of octadecyl rhodamine (R18). Although pH dependent fusion of R18-labeled PMV with cells expressing VSV G or influenza hemagglutinin could readily be measured using spectrofluorometry, fusion of CD4-PMV with gp120-gp41 expressing cells was only observable by fluorescence microscopy. However, the CD4-PMV were highly efficient in blocking HIV-1 infection and HIV-lenv glycoprotein-induced syncytia formation. The inhibition of HIV-1 env-induced cell fusion by CD4-PMV derived from human (H-PMV) and non-human cells (N-PMV) expressing CD4 was 10-20 fold more efficient as compared to sCD4. However, N-PMV required higher doses and longer preincubation times than H-PMV for full inhibition of cell fusion. Moreover, only H-PMV irreversibly blocked cell fusion. Because of the presence of high amounts of CD4, as well as accessory molecules which may irreversibly interact with gp120-gp41, H-PMV are powerful agents for inhibition of initial steps of HIV-1 entry into cells.

#### **Publications:**

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- Puri A, Grimaldi S, Blumenthal R. Role of viral envelope sialic acid in membrane fusion mediated by the vesicular stomatitis virus envelope glycoprotein. *Biochemistry* 1992;31:10108-10113.
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- Dimitrov DS, Broder CC, Berger EA, Blumenthal R. Calcium ions are required for cell fusion mediated by the CD4-HIV-1 envelope glycoprotein interaction. *J Virol* 1993;67:1647-1652.
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- Herrmann A, Clague MJ, Blumenthal R. The role of target membrane structure in fusion with influenza virus: effect of modulating erythrocyte transbilayer phospholipid distribution. *Membrane Biochemistry* 1993;10:3-15.
- Puri A, Clague MJ, Schoch C, Blumenthal R. Kinetics of fusion of envelope viruses with cells. *Methods Enzymol* 1993;220:277-287.
- Morris SJ, Zimmerberg J, Sarkar DP, Blumenthal R. Kinetics of cell fusion mediated by viral envelope proteins. *Methods Enzymol* 1993;221:42-58.

Schoch C, Blumenthal R. Role of the fusion peptide sequence in initial stages of influenza hemagglutinin-induced cell fusion. *J Biol Chem* 1993;268:9267-9274.

Bagai S, Puri A, Blumenthal R, Sarkar DP. Hemagglutinin-Neuraminidase enhances F protein-mediated membrane fusion of reconstituted Sendai viral envelopes with cells. *J Virol*, in press.

Herrmann A, Clague MJ, Blumenthal R. Enhancement of viral fusion by non-adsorbing polymers. *Biophys J*, in press.

Puri A, Krumbiegel M, Dimitrov DS, Blumenthal R. A new approach to measure fusion activity of cloned viral envelope proteins: fluorescence dequenching of octadecylrhodamine labeled plasma membrane vesicles fusing with cells expressing vesicular stomatitis virus glycoprotein. *Virology*, in press.

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

PROJECT NUMBER

201 CB 08320-18 LMMB

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October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Peptide Conformations and their Binding Sites

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Robert Jernigan, Ph.D. Deputy Chief, LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Kai-Li Ting, Ph.D. Computer Programmer LMMB, NCI

COOPERATING UNITS (IF ANY)

David Covell, FBSC, PRI, FCRDC

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.6

PROFESSIONAL:

0.6

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.)

The extremely large numbers of conformations, accessible to peptides of size 10 to 20 residues, make them difficult to consider both theoretically and experimentally. Complete sets of their conformations are impossibly large to generate. A new approach to combine calculations with specific constraints has been developed. The constraining information could originate either from experiment or from a molecular model with specified interactions. This approach for considering random peptide conformations with constraints has been formulated in the context of helix-coil theory.

Protein surfaces are being examined in order to develop methods to specify likely binding sites for peptides. Lattice fits of the target protein are extended to define an exterior shell. Each of the shell points is probed to evaluate the hydrophobicity of the protein residues in its neighborhood. The binding sites in 23 co-crystals of peptides bound to proteins are correctly identified by this procedure.

## PROJECT DESCRIPTION

**Major Findings:**

In most molecular calculations on large structures, an initial model or structure is required, and calculations typically refine this starting structure. It is interesting to develop methods that can perform larger conformational searches based more directly either on model constraints or on experimental data. An example of such data might be a salt bridge in an alpha helix which could be either postulated or based on experiment. We have developed a formalism for performing calculations of molecular conformation with such limited information. This approach might permit a crude determination of the relative feasibilities of several alternative models at an earlier stage in the development of a molecular model. The aim has been to bridge the gap between non-quantitative molecular model building and more rigorous but less directed molecular calculations. An application was made to salt bridges in the C peptide of ribonuclease A.

Known structures with bound peptides have been studied intensively in order to develop ways to specify hot spots for peptide binding. This new approach is quite successful when compared to known structures. The result is a favorable cluster of points immediately exterior to the protein. In a next stage of developing calculation methods, the best amino acids to place upon these points will be determined in a project aiming at inhibitor design.

**Publications:**

Jacchieri S, Jernigan RL. Variable ranges of interactions in polypeptide conformations with a method to complement molecular modeling. *Biopolymers* 1992;32:1327-1388.

Sumner SCJ, Jiang SP, Jernigan RL, Ferretti, JA. Conformational analysis of receptor selective tachykinin analogs: Senktide and septide. *J Biomol Str Dyn* 1992;10:429-439.

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

PROJECT NUMBER

Z01 CB 08363-11 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Membrane Protein Modelling

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

H. Robert Guy, Ph.D. Research Biologist LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Stewart Durell, Ph.D. IRTA Fellow LMMB, NCI

COOPERATING UNITS (IF ANY)

Harvey Pollard, NIDDK; Michael Zasloff, University of Pennsylvania.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NNCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

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.)

The primary goals of this project are to develop methods to predict structures of membrane proteins from their sequences and available experimental data, to use these methods to develop structural models of specific membrane proteins, and to work with experimental groups to test these models. We have developed a hierarchical approach to modeling membrane proteins. In the first phase we predict which segments cross the membrane and the secondary structure of these segments, in the second phase we predict the relative positions and orientations of the transmembrane segments, and in the third phase we use computer graphics and molecular mechanics energy calculations to produce models that predict positions of all atoms in the regions of the proteins that are modeled. We have developed models through the third phase for members of the following families of proteins: delta lysin, magainins, cecropins, alamethicin, pardaxin, annexins, and voltage-gated, calcium-gated, and inward rectifying potassium channels. Most of our time during the past year was spent developing models of the potassium channels. We have now established collaborations to begin the fourth phase of modeling of the potassium channel in which energetic factors such as water, lipids, ions, membrane voltages, and entropy are included in the energy calculations and the fifth phase in which functional properties such as gating, ion permeation, and drug binding mechanism are modeled. As part of our effort to improve energy calculations, Stewart Durell has used molecular dynamics simulations to study properties of macromolecules in aqueous solutions.

We have continued our collaboration with Michael Zasloff's group on antimicrobial molecules by developing models of a steroid-like molecule from shark called Squalamine and a polymyxin-like peptide from bullfrog called Ranalexin. We also have worked with Harvey Pollard's group to develop models of ion channels formed by Amyloid beta protein, which is postulated to cause Alzheimer's disease.

**PROJECT DESCRIPTION****Major Findings:**

Numerous experimental groups have continued to obtain data that confirm our models of the general folding patterns of voltage-gated potassium and sodium channel proteins and our hypotheses about which protein segments are responsible for ion selectivity, channel gating, and drug and toxin binding. Most of these experiments involve mutagenesis. Sequences of many more experiments involve mutagenesis. Sequences of many more homologous proteins have also been determined. These new data have led us to revise our previous detailed three-dimensional models of voltage-gated potassium channels and developed new detailed models of calcium-gated and inward-rectifying potassium channels. We have developed these new models so that the same general protein folding patterns can satisfy our modeling criteria for these three distantly related protein families and so that the structure of portions of these proteins postulated to determine ion selectivity is very similar for all three families of potassium channels. A major difference of these models from those developed previously is the structure of the ion selective segments; in our new models the lining of the narrowest portion of the pore is formed by the backbone segment. Four of these segments, one from each subunit, are surrounded by four alpha helices that precede the lining segments in the sequence. These helices, which span the outer two thirds of the membrane, are surrounded by additional alpha helices that span the entire membrane. These new models are more consistent with experimental results and satisfy our modeling criteria better than previously developed models.

Harvey Pollard's group in NIDDK have found that the protein postulated to cause Alzheimer's disease, amyloid beta protein, forms cation channels in membranes. They have suggested that channel formation may be the mechanism by which this protein kills nerve cells. In collaboration with them, we have developed three dimensional models of how this small protein could form a channel assembly. In these models, a relatively polar beta barrel, similar to that of porin channels, is surrounded by hydrophobic alpha helices. Experiments have been designed to test this model.

The atomic structure of the newly discovered antimicrobial agents Squalamine and Ranalexin differ dramatically from the Magainin and Cecropin antimicrobial peptides that we have modeled previously. Nonetheless, our modeling suggests that they may act in a similar manner; i.e., by interacting with membrane lipids and by possibly forming ion channels.

**Publications:**

Durell SR, Raghunathan G, Guy HR. Modeling the ion channel structure of cecropin. *Biophys J* 1992;63:1623-1631.

Cruciani RA, Barker JL, Durell SR, Raghunathan G, Guy HR, Zasloff M, Stanley EF. Magainin 2: A natural antibiotic from frog skin forms ion channels in lipid bilayer membranes. Eur J Pharmacol 1992;226:287-296.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
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Z01 CB 08370-10 LMMB

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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. Deputy Chief, LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Kai-Li Ting, Ph.D.	Computer Programmer	LMMB, NCI
Peter Greif, M.D.	Computer Prog. Anal.	LMMB, NCI
Vladimir Frecer, Ph.D.	Oncology Research Fellow	LMMB, 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 STAFF YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

0.0

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- (a1) Minors
- (a2) Interviews

B

SUMMARY OF WORK (USE STANDARD UNREDUCED TYPE. DO NOT EXCEED THE SPACE PROVIDED.)

A novel approach has been taken to the problem of protein folding that examines the complete range of accessible folded topologies. The first stage is to generate all possible shapes for a protein of given size and composition. The second procedure is to generate all conformations, with volume exclusion, upon a lattice in a space restricted to the individual compact space. The advantage of this two stage approach is that there is a high efficiency for conformation generation when there are exactly the same number of points as there are residues. The present studies have aimed at a more thorough evaluation of protein folds, with less than atomic detail. The assumption here is that the overall chain tracing is more important than the precise positioning of each atom. Such atomless structures can be evaluated with potential functions that resemble pairwise residue-residue hydrophobicities. These residue-residue potentials are being extended to include repulsive terms appropriate for packing considerations.

## PROJECT DESCRIPTION

Major Findings:

A principal goal of molecular biology is to understand the bases of molecular and biological recognition. An ultimate goal for theory in this area remains the calculation of favored macromolecular conformations directly from their sequences. Although we do utilize detailed atom-atom calculations, we feel that 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 has an ultimate aim of treating larger, more complex structures.

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 equivalents of water molecules. These derived contact energies follow intuition, with the most frequently and hence favorably interacting pairs being hydrophobic residues. These attractive potential energies have the character of a pairwise hydrophobicity index. The values reflect the actual situation inside of known proteins and provide a tool that can be applied to a variety of problems and, can be used in a simple way to assess the relative overall quality of different conformations.

These potential functions have been combined with frequencies of base substitutions to derive an amino acid substitution matrix. There is an excellent correlation between these values and the Dayhoff substitution matrix that was derived, not from structures, but from sequences of closely related proteins.

The present examples of generating all possible compact conformations on lattices indicate 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 would then permit detailed examination of local packing arrangements that favor interactions of side-chains within the protein's interior. By using this coarse-grained approach for examining conformational space and by subsequently adding atomic details onto this model, it should become possible to examine the role of amino acid sequence on three-dimensional structure.

Generalizing the approach so that an unknown structure can be considered as being approached by an initial generation of potential shapes for a protein of given size and composition. Then, all conformations are generated for each of these shapes, in large scale calculations.

**Publications:**

Jernigan, RL, Covell DG. Coarse graining conformations: a peptide binding example. In: Beveridge DL, Lavery R, eds., Theoretical biochemistry and molecular biophysics. Proteins 1991;2:69-76.

Jernigan RL. Generating general shapes and conformation with regular lattices, for compact proteins. In: Sarma, RH, Sarma MH, eds. Proceedings of seventh conversation in biostereodynamics. Structure and function 1991;2:169-182.

Miyazawa, S, Jernigan, RL. A new substitution matrix for protein sequence searches bases on contact frequencies in protein structures. Protein Engineering, in press.

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

PROJECT NUMBER

201 CB 08371-10 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Conformational Variation of DNA and DNA-Protein Binding

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Robert Jernigan, Ph.D. Deputy Chief, LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Gopalan Raghunathan, Ph.D.	Visiting Scientist	LMMB, NCI
Victor Zhurkin, Ph.D.	Visiting Scientist	LMMB, NCI
Shou-ping Jiang, Ph.D.	Visiting Scientist	LMMB, NCI
Brooke Lustig, Ph.D.	IRTA Fellow	LMMB, NCI
Kai-Li Ting, Ph.D.	Computer Programmer	LMMB, NCI

COOPERATING UNITS (IF ANY)

Dr. Jacob Mazur, PRI, Frederick, MD; Akinori Sarai, RIKEN Institute; Daniel Camerini-Otero, NIDDK; H. Todd Miles, NIDDK; V. Sasisekharan, NIDDK.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.2

PROFESSIONAL:

4.2

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.)

Conformational analyses of DNA show that, in addition to sequence specific preferences for mean positions of stacking, there are also substantial sequence specific dependencies in the conformational fluctuations. Both effects manifest themselves in the bending behavior. Consideration of the fluctuations was shown to be especially important for sequences with only small intrinsic static bends. Good agreement was shown between calculations of groove widths for such ensembles of helical forms and the reported hydroxyl radical cutting data.

Ways to treat RNA folding in three dimensions were considered. Transfer RNA was used as a test molecule to investigate large numbers of possible arrangements. In the largest generation of such conformations, over 2 million, several types of variant conformations were observed. There was some flexibility in the anticodon loop and several cases of "slip pairing with a single base bulge".

Triple helices of several kinds are being investigated. A highly symmetric triple helix structure, with three identical backbones, has been proposed based on fiber X-ray and molecular modelling. Another type of triple helix has been proposed as an intermediate for DNA recombination. It has unusual molecular features such that base triplets are isomorphic, and the helix is sufficiently elongated to weaken interactions between neighboring triplets, thereby circumventing non-specific interactions.

## PROJECT DESCRIPTION

### Major Findings:

A static picture of DNA double helical conformations is only a first approximation. In addition there can be substantial flexibility because of the fluctuations about the mean static form. Some sequences such as runs of A's show some significant static bend at each base step with relatively little fluctuation; whereas others such as pyrimidine-purine steps manifest a large flexibility. Methods for calculating these properties have been refined. Notably good agreement has been shown between the model that includes both modes of bending and electrophoresis and hydroxyl radical cutting experiments (Zhurkin, Mazur, and Jernigan).

Conformations of the 76 nucleotide phenylalanine t-RNA have been studied. Different chain conformations have been placed on the coordinate points of this structure by placing alternative chain tracings upon the various points of this structure. The large number of proximate bases that can form base pairs prevents the direct generation of the combinations of all possible base pairs. It was found that the use of secondary structures was necessary to bring the calculations into feasible range. After a set of secondary structures were found, all combinations of the remaining potential tertiary base pairs were generated to obtain, in some cases, more than 2 million conformations of this small t-RNA. The number of conformations for even so restricted a situation was surprisingly large and implies that general approaches to RNA folding will require preliminary reductions in conformations, such as secondary structure approaches (Lustig and Jernigan).

Triple helix models are being developed for both the case where the third strand is parallel and anti-parallel to its identical strand. A new highly symmetric triple helix (H-form) has been proposed on the basis of fiber X-ray and molecular modelling (Ragunathan, Sasisekharan, and Miles). Models for protein-nucleic acid interactions have been proposed, including that with rec A, for recombination. The experimental data indicate that when recombination proteins join a DNA duplex with a single stranded DNA, they form a triple helix (R-form DNA). On the basis of the conformational energy minimization, we have built the stereochemically feasible model of the R-form which is consistent with the chemical modification data for deproteinized DNA, obtained in the Laboratory of R.D. Camerini-Otero (NIDDK, NIH). Unlike the well known non-enzymatic triplexes, in R-form the third, R-strand, is parallel to the identical duplex strand. This is the first triple helix shown to be sterically possible for any arbitrary sequence. The biological significance of R-form is that it secures the stringent recognition of the homologous chromosomes in the course of meiosis, and facilitates the strand exchange (Zhurkin, Ragunathan, Camerini-Otero, and Jernigan).

The stabilization of nucleic acid structures through interactions with specific ions is being investigated. Quadruple stranded structures show selective

stabilization for various monovalent ions. Stable forms appear to have strongly localized ionic binding sites (Jiang and Jernigan).

Publications:

Howard FB, Miles HT, Liu K, Frazier J, Raghunathan G, Sasisekharan V. Structure of dTndAndTn: The DNA triple helix has B-form geometry with C2'-endo sugar pucker. *Biochemistry* 1992;31:10671-10677.

Raghunathan G, Miles HT, Sasisekharan V. Symmetry and molecular structure of a DNA triple helix:dTndAndTn. *Biochemistry* 1992;32:455-462.

Ulyanov NB, Gorin AA, Zhurkin VB, Chen BC, Sarma MH, Sarma RH. Systematic study of nuclear overhauser effects vis-a-vis local helical parameters, sugar puckers, and glycosidic torsions in B-DNA: Insensitivity of NOE to local transitions in B-DNA oligonucleotides due to internal structural compensations. *Biochemistry* 1992;31:3918-3930.

Olson WA, Marky NL, Jernigan RL, Zhurkin VB. Influence of fluctuations on DNA curvature. A comparison of flexible and static wedge models of intrinsically bent DNA. *J Mol Biol* 1993; 232: in press.

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Barber AM, Zhurkin VB, Adhya S. CRP binding sites. Evidence for two structural classes with 6-bp and 8-bp spacers. *Gene* 1993; in press.

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

PROJECT NUMBER

201 CB 08380-09 LMME

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Molecular Structure of Animal Viruses and Cells by Computational Analysis

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Jacob V. Maizel, Jr., Ph.D. Chief, Laboratory of Mathematical Biology, LMME, NCI

OTHER PROFESSIONAL PERSONNEL:

John Owens	Computer Programmer Analyst	LMME, NCI
Shu-Yun Le	Visiting Fellow	LMME, NCI
Lewis Lipkin, M.D.	Medical Officer	LMME, NCI
Shou Liang Lin, Ph.D.	Senior Staff Fellow	LMME, NCI
Chung-Jung Tsai, Ph.D	Visiting Fellow	LMME, NCI
Ann Barber, M.D.	Guest Researcher	LMME, NCI

COOPERATING UNITS (IF ANY)

Dr. Danielle Konings, Univ. of Colorado, Boulder, CO; Dr. Hugo Martinez, Consultant, PRI, NCI/FCRDC, Frederick, MD; Dr. Nahum Sonenberg, McGill Univ., Montreal, Canada; Edward Chu, NMOB, NCI; Drs Daniel Fischer and Haim Wolfson, Tel Aviv Univ., Israel.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892 and Frederick, MD 21702-1201

TOTAL STAFF 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.)

Complex macromolecular processes and the structural organization of normal, infected and transformed cells are modeled using viral systems. Computers are used to study nucleic acid and protein sequences that embody the information of living systems.

Computer analyses of proteins and nucleic acids are developed and implemented in conjunction with techniques of biochemistry, virology, and electron microscopy on sequences of picornaviruses, adenoviruses, and 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 vector and massively parallel supercomputers, minicomputers 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.

## Other Professional Personnel (continued):

Kathleen Currey, M.D.	Guest Researcher	LMMB, NCI
Ruth Nussinov, Ph.D.	Guest Researcher	LMMB, NCI

## PROJECT DESCRIPTION

Major Findings:

**RNA secondary structure:** RNA structure plays an increasingly important part in the active functions of organisms. New discoveries of catalytic activities and specific binding to non-nucleic acid molecules add to the interest. There is demand for methods and results from computational structure prediction to augment the results of experimental structure determination, which are not as extensive as for proteins. As the power of computers and algorithms increase the importance of structure prediction grows. Predicted models aid in understanding experimental data on the intricacies of gene expression, and in design of new experiments. A number of biotechnology firms are pursuing pharmaceuticals and other products based on RNA structures and activities.

Our continuing research on RNA structure prediction focuses on improving the power of predictive methods and applying them to problems in important biological systems. "Unusual folding regions" (UFR) are defined as sub-sequences of nucleic acid that are predicted, using the best available rules, to either higher or lower stability than would a random sequence with the same base composition. Two programs, SEGFOLD and SIGSTB, improved to include the latest energy rules and to provide greater ease of use were applied to viral and cellular sequences. (Le, Chen) An algorithm based on combination of energy rules and phylogenetic comparison for locating common features among homologous sequences is under development.

Structural motifs were detected in the 5' untranslated regions of several animal viruses (e.g., picornavirus, infectious bronchitis virus, and pestivirus genomes) and in a proto-oncogene (p53). These structures are candidates as indispensable elements for internal translational initiation in eukaryotic cell and virus systems. (Le, Sonenberg(McGill U)

In translation of gene 60 of bacteriophage T4, ribosomes bypass a 50-nt gap sequence. We observe a structural motif that is postulated to be involved in the ribosomal jumping.

Chu and colleagues (NCI-Navy Medical Oncology Branch) showed that in treatment of colorectal cancer with 5-fluorouracil there is an increase in expression of the target enzyme, thymidylate synthetase (TS), leading to resistance to the drug. Expression is regulated at the level of TS mRNA translation, with TS interacting directly with its mRNA. In initial studies we predict interesting structures in the RNA. Work is continuing on human, *E. coli*, and bacteriophage



TS mRNAs and the human C-mys system to enhance the understanding and treatment of this important cancer.

**RNA 3-D structure prediction:** The goal of structure prediction includes developing methods for 3-dimensional structure that will give models depicting affinity binding and catalysis at the atomic level. A novel approach that starts with a predicted secondary structure model and produces a 3-D model by twisting the planar model into A-form double helices and coiled loops as a first approximation. Co-axial stacking of stems, formation of pseudoknots and maintenance of acceptable geometries are done automatically. Interactive guidance using pseudobonds can be provided to bring unpaired, short complementary segments of loops together while providing reasonable bending at acceptable points in the structure. All the bonded atoms of nucleotide subunits and many atoms in non-bonded substructures are placed at correct distances. Three-dimensional coordinates can be written to files that are suitable for conventional molecular modeling programs, such as MIDAS, QUANTA, INSIGHT and others, in which refinement by energy minimization using classical potentials can be done. These models may be re-introduced into the folding program for convenient large-scale modification. (Martinez, Konings, Le).

**Protein 3-D structure comparisons:** A novel project involves developing and applying a computer vision and robotics based approach to 3D protein structure studies. It exploits a rotationally and translationally invariant representation of rigid objects (atomic coordinates of protein structures, or descriptors of molecular surfaces), resulting in a highly efficient, fully automated tool. These techniques are uniquely suitable to three dimensional, structural problems. Specifically, they have been applied to (i) comparisons of protein structures and searches of substructural motifs and to (ii) docking of complementary substructural surfaces. (i) Given the three-dimensional coordinate data of the protein structures to be compared, the method developed by Nussinov and Wolfson automatically identifies every region of structural similarity between the structures without prior knowledge of an initial alignment. The technique is extremely fast, template free and sequence-order independent. There are three novel aspects in this method. First, it compares the three dimensional structures of proteins completely regardless of the order of the residues in the chain. This allows detecting similarities between protein molecules whether these are on their surfaces or in their interior. The algorithm views atoms as collections of unconnected points in space. Spatial similarity between isolated atoms (residues) is thus obtained regardless of insertions, deletions and chain directionality. Second, recurring substructural, "real" 3-D motifs, are detected in a set of structures without a prior predefinition of the motifs. Furthermore, all molecules in the database can be compared simultaneously. Third, the method is extremely fast, with a typical running time of less than 3 seconds for a comparison between two proteins on a SG workstation, or 8 minutes for a comparison of one protein against a representative set of proteins from the crystallographic database, consisting of 170 protein structures.

The method has been used to compare a trypsin-like serine protease, beta-trypsin, against the crystallographic database. Besides detecting homologous trypsin-like proteases, the results automatically identify the similarities of the active site of beta trypsin with the active sites of subtilisin-like and sulfhydryl proteases and with the core protein of the sindbis virus. The method has also been applied to the search and identification of recurring, non-linear, 3-D motifs within alpha/beta protein domains. Geometrically equivalent, out-of-sequential order structural elements which could not have been obtained by other techniques have been achieved. An extension of the method is applied to searches of motifs on the surfaces of proteins. This method enables routine comparisons of any new structure - whether determined crystallographically, by NMR or computationally - against the database of 3-D structures, much in the same manner as today investigators compare a newly determined protein or DNA sequence with the sequence database. We plan on further developing, optimizing and applying this technique to searches of 3-D motifs in the structural database. Detection and cataloging of such motifs is expected to result in enumeration of 3-D units of protein-folds, yielding an insight into protein stability and packing.

The proposed procedures to accomplish this goal, at this stage, can be divided into three steps. First, a pairwise protein structure comparison based on only the  $C_{\alpha}$  backbones should give complete, non-redundant matched pairs for an analysis in the next step. Among matched pairs, those with similar transformation matrices used to superimpose a pair of proteins are considered as redundant. In addition, two matched pairs, even with distinct transformation matrices but having the identity number greater than a threshold value, should also be considered redundant. Second, in a series of one-against-all (a representative collections from protein data bank) comparisons, all of the known secondary structures (for example,  $\alpha$ -helix and  $\beta$ -strand), known folding units (helix-turn-helix, Greek key, and  $\beta$ -barrel motifs), even recurring spatial arrangements (active sites), and maybe new 3-D motifs, are to be automatically extracted and classified by means of a statistical analysis employing those non-redundant matched pairs. This is the main part of this project. Third, based on the analyzed information obtained in the previous step, a new protein structure, will be given a score which summarizes its resemblance to the known native protein conformation by an one-against-all comparison run. Besides, an associated graphic representation will give the summarized matches of the examined protein with the proteins in the databank (Tsai, Nussinov).

(ii) The second project within this framework in the Office of the Chief involves developing and applying the computer vision and robotics based techniques to the problem of docking a ligand onto a receptor surface. The association of proteins with their ligands involves intricate inter- and intramolecular interactions, solvation effects and conformational changes. In view of such complexity, a comprehensive and efficient approach to predicting the formation of protein-ligand complexes from their unbound components is not yet available. The approach we have developed is purely geometric. It is

general, and assumes only knowledge of the coordinates of the two molecules. No biological information about the binding sites is incorporated.

There are two criteria to the success of such a general method. The first requires that the method be very efficient. The second requirement demands that the number of geometrically correct solutions obtained be relatively small, since each would need to undergo detailed, time consuming, energy calculations. Our method fulfills these requirements. Starting with the entire surfaces with the two molecules, geometrically correct solutions are obtained in very short times. Complete protein-protein docking solutions are achieved in under 10 min on a SG workstation. The number of solutions obtained is small too, either in the tens or in the hundreds. For protein-small molecule docking, correct solutions are obtained in under a minute. We are able to achieve this high level of performance by incorporating our novel surface representation, in which the molecular surface is covered by a set of points sparsely disposed at strategic spots. The representation is accurate (represents the true shape of the surface,) complete (covers the whole molecule,) concise (only a few points per surface atom,) rich in describing local surface properties (surface normal, area, curvature, connectivity, etc.). Comparing to the most commonly used surface representations, this representation reduces substantially the required number of points, and hence the costs of their applications which often depend on the number quadratically or higher. The representation also allows employing more definite and accurate surface properties. For example, surface normals, which are important in describing surface directionality, haven't been fully employed in docking problems until now. The representation is also expected to be useful in fast visualization of molecules. Matches of e.g., caps (pits) of the receptor with pits (caps) of the ligand are sought. Only after a "significantly large" match between the receptor and the ligand has been detected, the least squares fitting and the transformation of one molecule with respect to the other which achieves it is computed. Minimization of the energy will be carried out next. This step is critical in order to find the optimally docked receptor-ligand conformations (Lin, Nussinov).

Other related projects which are (iii) adaptation of Computer Vision based approaches to refinement of NMR structures by mapping the obtained inter-proton distances onto the crystallographically determined structures. In addition, (iv) robotics-based techniques have been designed and are implemented for the docking of ligands into receptors, allowing flexibility about molecular "hinges". Hinges may be selected between atoms in drugs or between domains in proteins. Preliminary results using this approach have been very encouraging.

**Protein and nucleic acid sequence comparisons and motif detection:**  
The general purpose DNA/protein database search and sequence retrieval programs, Gnsrch and Dsrch have been combined into one program and rewritten in the "C" programming language. The new program can run on any VAX/VMS system or any of the Laboratory's Unix systems able to access the BSC mass-storage system. The "C" version execution times are noticeably faster than previous

Pascal versions and both the database files and index files are 30-40% smaller than previous. Gnsrch can create sequence libraries or extract annotation text, feature table information, or any combination of the above, based on search criteria of nearly any word, author name, accession number, etc. that can be found in the database files. Additional functions were added to translate DNA coding regions to protein sequences and create more flexible output options. In the past, primary interest in this program has been its ability retrieve and convert sequences into a formatted library that is compatible with one of the popular sequence comparison or secondary structure programs. More recently, copies of the program have been distributed to Merck and the Hebrew University of Jerusalem specifically to utilize the program's ability to retrieve sequence information based on the GenBank features table. Gnsrch is the only known program of its nature able to correctly assemble a sequence or coding region based on the "relational database join" information contained in the widely distributed GenBank flat file format.

The fixed-point alignment program, Feature, has been recoded in the "C" programming language to take full advantage of the VAX and Unix environments and the new database index structure created by Gnsrch. The fixed-point alignment methodology proposes a specific sequence segment or pattern to act as an alignment guide or zero alignment point of a sequence. Other sequence segments or patterns are then selected to be listed relative to a "downstream" or "upstream" position to the zero alignment point. Use of fixed-point alignment methodology in the past has been a primary tool for the study and discovery of significant RNA signals, alignment of protein motifs, and the study of recurring, periodic patterns in DNA sequences by members of the Laboratory and visiting scientists. To date well over a dozen publications have been wholly or substantially based on data generated by one of the versions of the Feature program. The original Feature data plots have been removed from both the Tektronix and the Symbolics 3650, Common Lisp environment and been rewritten as precoded PostScript modules that are inserted into a reformatted Feature data file as needed. Plots may then be generated on any available PostScript printer. The PostScript modules developed have been used in other programs to create X-Y plots from data that were either printed locally or sent to collaborators via electronic mail or diskette.

Programs created to study specific motif patterns and recurring patterns in large collections of sequences in collaboration with Andreas Konopka produced a number of papers and a book chapter on specific applications of this phenomenon with Dr. Konopka as senior or sole author. An application has been substantially completed that can be used as a general tool to study shorter-length motifs in sequence libraries. Output from this general version is an all-way sequence comparison ( $N^2$  matrix) of motif counts that occur exclusively in one, two, or at a selected cutoff level if a motif occurs in more than two sequences. This program should find application among investigators wanting to do a preliminary "shot-gun" analysis of possible signals, or create a sequence library with members that always contain or lack common motifs of a specific size or sequence pattern (Owens).

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

PROJECT NUMBER  
 Z01 CB 08381-10 LMMB

**PERIOD COVERED**

October 1, 1992 to September 30, 1993

**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, LMMB, NCI

**OTHER PROFESSIONAL PERSONNEL:**

Yecheng Wu, Ph.D.                      Special Volunteer & CSPI (CRADA)                      IPS, LMMB, NCI  
 Kyle Upton                      Scientific Applications Analyst                      PRI/FCRDC

**COOPERATING UNITS (IF ANY)**

Dr. J. Myrick, CDC/Atlanta; Dr. C. Merrill, NIMH; Dr. P. Rogan, Penn State Med. Sch.;  
 Dr. T. Krekling, Agr. Univ. Norway; Dr. R. Leimgrubber, Monsanto Co.

**LAB/BRANCH**

Laboratory of Mathematical Biology

**SECTION**

Image Processing Section

**INSTITUTE AND LOCATION**

Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

**TOTAL STAFF YEARS:**

1.5

**PROFESSIONAL:**

1.5

**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.)**

Analytic methods are continuing to be developed and applied using the GELLAB-II software system - an exploratory data analysis system for the analysis of sets of 2D electrophoretic gel images. It incorporates sophisticated subsystems for image acquisition, processing, database manipulation, graphics and 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 and on workstations over wide area networks. Substantive GELLAB-II applications include: Ongoing studies of cadmium toxicity in urine (Myrick), Rett syndrome (Myrick), Vermont mercury study (Myrick), serum dioxin study (Myrick), fetal alcohol syndrome study (Myrick) - a new study will be neural tube defects (Myrick); 2D DNA gels for identifying differentially expressed genes (Rogan); investigation of radioactive accidents and fall-out from Chernobyl (Krekling).

In a collaboration with Merrill, we are developing a relational 2D gel interactive disease-protein-spot database system for answering queries relating proteins in plasma, serum, urine and CSF as a diagnostic tool. We continue to explore image conferencing of 2D gel images as well as other problem domains using Xconf.

GELLAB-II has been exported to and is being supported at: CDC/Atlanta (Myrick), U. Norway (Krekling), Monsanto Research Labs (Leimgrubber et al.). This year, CDC has been the primary Beta test site for GELLAB-II. Additional changes to help continued collaboration with and exporting of enhanced graphical-interface versions of GELLAB has been actively pursued and is the primary activity this year.

## PROJECT DESCRIPTION

### Major Findings:

Much of this year was spent in the technology transfer of our GELLAB-II system to CSPI (CRADA). They are in the process of commercializing it with GELLAB-II+ which will run on less expensive Windows-NT PCs. Two-dimensional gel electrophoresis analytic methods are continuing to be developed, enhanced and applied using the GELLAB-II system. Further effort was made with our CRADA partner in further simplifying and enhancing the user interface for GELLAB-II programs. A new fast 2D gel spot finding and quantitation algorithm was developed that is more suitable for very high resolution gel images with larger 16-bit pixel grayscale resolution that is more suitable for the new class of phosphor-imaging scannerings. A new database storage scheme, Paged Indexed Buckets, was developed to minimize disk space requirements without significant access penalties. Both of these improvements will make it easier to port the technology to inexpensive PC systems.

These new graphical interfaces make learning and using the system much easier.

The GELLAB-II programs have grown to over 250,000 lines of code and continues to be fine tuned to simplify the user interface and analysis.

We have successfully negotiated and entered into a CRADA with CSPI with the help of OTD/NCI to transfer the GELLAB-II technology to private industry so it can be commercialized. During the first phase, Dr. Yecheng Wu (CSPI) worked in our laboratory for 6 months to learn about, transfer and help improve the GELLAB-II technology. We are currently in the second phase where Dr. Wu and others back at CSPI are turning GELLAB-II into a more marketable and lower cost GELLAB-II+ system for a PC running Windows-NT. Such commercialization will result in wider use and better support of the GELLAB-II technology than we can provide.

This year, Kyle Upton (PRI/FCRDC) worked on several graphical user interface (GUI) subsystems of GELLAB-II. Part of this effort was in developing a general purpose GUI toolkit library, LIBW, which we then used in many other parts of the system to give the interface a consistent look and feel. Using LIBW, we reimplemented the data acquisition systems GETAC11 for accessioning gels from various scanner sources and LANDMARK11 for interactively defining and editing landmarks necessary for spot pairing. We also rewrote the general image input library so that we can read images from a variety of image sources and pixel depths making the system more portable and usable with the new class of phosphor-imaging gel laser scanners. He is also rewriting the general image viewer XPIX used by GELLAB-II to use these new libraries and also a spot editor for use with GELLAB-II.

The LIBW interface was then used in building the new GUI interface to the menu driven composite gel database system CGELP2. This makes it much easier to use



since users don't have to remember commands but instead can just use pull-down menus and direct manipulation by pointing to spots in the database through a dynamic-Rmap image.

Although our spot segmentation algorithm has worked well especially with some of the recent improvements including spot merging and splitting, it requires considerable CPU time for large high resolution images. This is because it uses a dual stack based algorithm to propagate a spot's boundary with computations greater than  $\text{Order}(n*n)$  ( $n$  the area of the spot). We modified our basic algorithm to use a more efficient parallel algorithm that can result in major improvements in processing time (roughly 10X faster for the new method) and is reported in the paper by Wu et al. 1993. This speedup was essential because one of our goals is to be able to have the investigator interactively titrate segmentation parameters while sitting at a workstation.

We developed a new 2D disk data based structure called Paged Indexed Buckets that optimizes both retrieval times and disk space for composite gel database files. The new method is several times more efficient in disk space while having similar retrieval speed to the old method. Fast access of 2D gel databases is important for rapid searching for protein differences between sets of gels from an experiment. The GELLAB-II system organizes corresponding spots into Rspot sets that index fixed regions in the Paged Composite Gel DataBase file. This is adequate for an existing database, but has several problems.

(1) Building the initial database requires guessing how much disk space to pre-allocate for each corresponding spot (i.e., spots from different gels). If it ever runs out of pre-allocated space during this process, it expands the size of each corresponding set of spots and copies the old database data into the new in-place on the disk. (2) In editing, if a new spot is created, the system can also go into this expansion mode. The time spent can be appreciable - depending on the size of the database (order of 100 gel database). (3) Because each set of corresponding spots is the same size, we waste space in most Rspot sets since they do not require the additional space a few Rspot sets require. The main implication of this change is that spot editing can take place in the composite gel database without any disk access penalties in wasted disk space or access time. This algorithm is reported in Lemkin et al. 1993.

#### Protein-disease relational database system

We have developed a GUI based prototype, GELQA, for the protein-disease relational database project with Carl Merrill's group (see below). The prototype has shown the feasibility of the concept and is helping us iterate toward the final design.

#### Xconf Image Conferencing

We have continued our research on using the Xconf image-conferencing system to aid collaborative research. Xconf was originally developed to explore remote collaboration of 2D gel images using workstations connected via national and

international networks. Conferees may interactively point to and discuss image data with actions visible to everyone in the conference. This year we are beginning to investigate using image conferencing in other problem domains with other types of images.

### Collaborations

Our primary collaboration this year has been with Dr. James Myrick (CDC/Atlanta). In addition to being our major biomedical collaborator he has been invaluable in beta-testing the GELLAB-II software. We have increased our collaboration by investigating several large 2D gel databases focusing on a cadmium toxicity study (29 gels); a Rett syndrome study (77 gels); fetal alcohol syndrome (52 gels); as well as studies of Mercury (86 gels) in urine and serum dioxin. We have also been able to experiment with image-networking using the Xconf over the Internet to share data. As a result we will be making changes in the Xconf protocols to make network based collaboration easier.

The six 2D gel CDC studies include: (1) Denver Cadmium Study. This is a project involving NIOSH and CDC to determine the health effects of occupational cadmium exposure in a group of metal recovery plant workers. The Protein Electrophoresis Laboratory (Clin. Biochem. Br., Div. Env. Health Lab. Sci., Nat. Center for Env. Health, CDC) has analyzed the urine samples from these workers with a BioImage system to attempt to find a better protein biomarker(s) of cadmium toxicity using statistical analysis. The project has been extended to include analysis of the same 29 gel images by the GELLAB-II system. Other studies include: (2) Rett Syndrome Study. Rett Syndrome is a unique mental retardation syndrome reported only in young girls (1:10,000) for which there are no known diagnostic markers. We are using quantitative 2D electrophoresis to analyze 12 sera from affected children and 10 age-matched controls to create a gel-to-gel matched database of detectable proteins. Statistical analysis of the database will be used to search for any protein(s) that may be of diagnostic significance for Rett Syndrome. (3) Vermont Mercury Study. Thermometer factory workers in Vermont are the subjects of this study. Twenty urine samples are being analyzed by 2D electrophoresis, and the intensities of matched proteins will be correlated with known body burdens of Hg to find an early marker of Hg toxicity. (4) A Serum Dioxin Study. This project involves persons with known body burdens of 2,3,7,8-TCDD (dioxin). Twenty sera are being analyzed by 2D, and matched, quantified proteins will be correlated with the serum dioxin concentrations. The health effects of low levels of dioxin are largely unknown in humans. Early serum protein changes may lead to further studies that could better describe the health effects from dioxin. (5) Fetal alcohol syndrome study. Twelve blood sera from children with FAS and eight control children are being analyzed with the GELLAB-II system to detect protein biomarker(s) of this syndrome that affects 1:700 live births in the US. FAS is totally preventable by abstinence during pregnancy, but an early marker of FAS is needed to better identify neonates. FAS and the less severe form, Fetal Alcohol Effects (FAE), are extremely difficult to diagnose early after birth. A serum biomarker will allow early intervention for the child and counseling of the mother for future pregnancies. (6) Neural tube defects. This new study

involves analyzing sera from mothers in South Carolina who gave birth to children with Neural Tube Defects. Proteins in sera that are unique to the case mothers, or vice versa, will be characterized to determine their tissue origins.

A collaboration has been started between the IPS and Dr. Carl Merrill's group in the Laboratory of Biochemical Genetics, NIMH. The goal of this collaboration is to create relational databases for body fluid proteins: plasma, spinal fluid and urine proteins during normal physiological and disease states. These databases will be linked to the high resolution 2D electrophoretic protein patterns that have been derived from each of these fluids.

A number of individuals have initiated databases concerned with proteins in body fluids. The Andersons pioneered this effort with their serum and plasma databases, and Goldman and Merrill with their cerebrospinal fluid database. Hochstrasser, is currently updating the plasma database. However, these databases are primarily concerned with the identification of the protein spots located on the high resolution 2D electrophoretograms. There has been no serious effort to relate a protein pattern database to disease states. This task of relating the protein database to disease states may be facilitated because there is a large amount of literature about plasma, serum, CSF and urine protein changes that have been determined as varying in physiological and disease states over the past two decades. While many of these protein changes were measured by techniques other than electrophoretic, that is by: enzymatic assays, or immune assays, or in some cases merely interaction with specific substances and sedimentation rates, these data can then be used as a basis for establishing a proteins' changes in disease states.

The primary question that will be asked of this database is: "if a specific set of proteins is changed, what disease does this relate to"? Conversely, one should be able to ask for a specific disease: "what proteins are predicted to be affected and by what order of magnitude"? For example, an investigator should be able to ask which proteins will be affected and in what manner if the patient has a specific disease, such as lupus erythematosus. This type of query may provide relationships between diseases which may have otherwise seemed unrelated. For example, there may be similar changes in schizophrenia and Alzheimer's disease which show up in this type of analysis, such as the increase in alpha2-haptoglobin which we have observed in spinal fluid in these diseases, and which would not have been uncovered by other approaches.

Despite all clinical tests that are performed in hospitals throughout this nation and others, little effort has been made to measure the relationship of multiple protein changes to disease states, despite the fact that such an approach would offer an increased amount of robust data for diagnosis of disease states.

Trygve Krekling (Agric. Univ. Norway) has collaborated in extending the range of images which GELLAB-II can interpret. His was the first site which we were able to directly access over the Internet and this helped us develop strategies

for updating user GELLAB-II systems with minimum effort. In addition, the Isotope and Electron Microscopy Laboratories are investigating the possibility of using GELLAB-II in ongoing investigations about localization and characterization of radioactive fall-out material from Chernobyl and elsewhere. Soil samples from polluted sites are deposited on cardboard with sticky surfaces and autoradiographs made. Autoradiographs are analyzed, using GELLAB-II, for localization of radioactive particles and estimating their activity. Based on this, pieces of the cardboard (containing active particles) are cut out and prepared for scanning electron microscopy and X-ray analysis for determination of micro-structure and element composition. These particles, air carried, are very small (micron - nanometer range), and would have been hard to isolate from the 10,000's of other particles using other methods. Particle size and micro-structure are important parameters about bio-availability since there is often an inverse relationship between size and bio-availability.

We are continuing with Peter Rogan (Penn State Med. Sch.) to develop new methods in GELLAB-II to help analyze 2D DNA gels. These 2D DNA gels are based on the use of restriction enzymes and can be used for monitoring active transcription sites in yeast and other systems. We have successfully demonstrated that the introduction of a foreign methylase into yeast cells can show cell-type specific sites of genomic modification as unique spots on a two-dimensional electrophoretic grid (published in 1991). A manuscript describing this technique, the biology of the methylation system, and these results are in preparation.

We have developed a technique designed to detect active transcription templates in eukaryotes. The E. coli dam methylase gene has been introduced and expressed in *Saccharomyces cerevisiae*. Active eukaryotic transcription templates can be enzymatically tagged by DNA methylase *in vivo*, whereas unexpressed genomic sequences fail to become methylated. Modified sites are detected by cleavage with restriction enzyme isoschizomers that exhibit differential sensitivities to methylation.

These digestions are carried out sequentially so that methylated and unmethylated fragments can be separated in a two-dimensional (2D) agarose gel matrix. Spots corresponding to methylated fragments are identified by hybridization with interspersed repetitive sequence probes. Autoradiographs are analyzed with GELLAB-II developed for data analysis of multiple two-dimensional images. The spot recognition algorithm of GELLAB-II was modified to improve detection of the diffuse spot shapes generated by this technique. We would like to adapt this technology for the analysis of human genomic sequences. The approach will be analogous to the experimental and analytical method developed in yeast.

In a new collaboration with Richard Leimgrubber and James Malone at Monsanto, we are investigating the use of GELLAB-II in constructing and searching toxicology 2D gel databases of liver cells and other cell lines. They will be creating a 2D gel database as part of a comprehensive toxicology program. Data

obtained from image analyses of 2D protein profiles of various cell types +/- treatments with various pharmacological agents will be used to address toxicity issues, among others. GELLAB-II will be used for helping identify unique induced proteins (possessing the desired activities) within the complicated mixture of cellular proteins. Correlated with this is the analyses of certain 2D gel profiles to study proteins that are coordinately regulated in response to some external agent.

**CRADA Information:**

CRADA Partner: CSPI, Billerica, Massachusetts; CACR-0115

Title: The Transfer and Commercialization of GELLAB-II 2D Electrophoretic Gel Analysis Software

Date CRADA initiated: July 30, 1992

Objectives: Transfer and commercialization of GELLAB-II 2D electrophoretic gel analysis software in order to get the technology into more research laboratories.

Methods employed: Optimize NCI UNIX X-Windows version and then convert it to Windows-NT for running on low cost popular PCs.

Significance to biomedical research: This work will help make the exploratory computer data analysis of 2D gels more available in research laboratories where it will be commercially supported.

**Publications:**

Lemkin PF. Xconf: a network-based image conferencing system. Comput Biomed Res 1992;1:1-27.

Lemkin PF, Myrick J, Upton K. Splitting merged spots in 2D page gel images. Applied and Theoretical Electrophoresis 1993;3:163-172.

Lemkin PF. Representations of protein patterns from 2D gel electrophoresis databases. In: Pickover C, ed. The visual display of biological information. Teaneck, New Jersey: World Scientific, in press.

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

PROJECT NUMBER

Z01 CB 08382-10 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Computer Analysis of Nucleic Acid Structure

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Bruce A. Shapiro, Ph.D. Computer Specialist IPS, LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Kathleen Currey, M.D.	Guest Researcher	LMMB, NCI
Morton L. Schultz	Electronics Engineer	LMMB, NCI
Maciej Czerwinski		LMC, NCI
Jacob V. Maizel, Jr., Ph.D.	Chief, Lab. of Math. Biol.	NCI
Joseph Navetta	Senior Applications Analyst	PRI/FCRDC
Wojciech Kasorzak	Senior Applications Analyst	PRI/FCRDC

COOPERATING UNITS (IF ANY)

Dr. Eric Baldwin, PRI; Dr. Dennis Shasha, Courant Institute of Math Sciences, NYU;  
 Dr. Jih-Hsiang Chen, PRI; Gary Smythers, PRI; Mark Gunnell, PRI.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

TOTAL STAFF YEARS:

2.0

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.)

Massively parallel computation has been a significant portion of the past year's research effort. The MasPar MP-1 has been upgraded to a 16,384 processor MP-2 with a 6 Gigaflop peak performance capability. Several algorithms have been developed and adapted to the MasPar including the newly developed genetic algorithm, a very fast version of the suboptimal dynamic programming algorithm for RNA structure prediction, a very fast and sensitive sequence comparison algorithm for determining sequence homologies in proteins and nucleic acids, and a visual docker for docking drugs with a protein substrate. In addition, work has continued on the heterogeneous RNA structure analysis system with improvements in its graphical presentation capabilities, RNA database matching facilities, mutated structure generation and extensions to the MasPar interface.

The above system in conjunction with gel shift experiments has been used to help determine the binding site of nucleocapsid protein NcP7 of HIV-1 and the RNA structural components that determine this site. This protein is important for encapsidation of the virus genome, RNA dimerization and primer tRNA annealing *in vitro*. The system is also being used to help determine the structure of human cytochrome mRNA which contains internal "inframe" UGA codons of unique dual function: termination of translation or insertion of selenocysteine. Unique RNA structures have been found in the 3' untranslated region of the molecule. A new algorithm for discovering motifs in protein sequences has been developed and is undergoing testing. It uses the concept of developing the motifs from a small sample of database sequences and then refining these motifs by running them against sequences in the database. Experimental results of running this algorithm on three protein families is giving good performance.

**PROJECT DESCRIPTION****Major Findings:**

During the past year massively parallel computation has become a significant part of the current research effort. We have been researching, testing and upgrading a massively parallel computer architecture for the Laboratory of Mathematical Biology and Frederick Biomedical Supercomputer Center. The acquisition and upgrade of a MasPar MP-1 to a MasPar MP-2 has changed the way many computational problems within our laboratory are being thought about. The MP-2 contains 16,384 processors and is capable of peak speeds of 6 gigaflops/second on single precision floating point. It is believed that massively parallel systems are the means by which grand challenge problems will eventually be solved and therefore is an essential element in the computational facilities of the laboratory. Several applications are actively being adapted to this architecture.

At the present time the first phase of research into a new class of algorithms, "genetic algorithms", for RNA folding is being completed (Joseph Navetta). This new algorithm is highly parallelizable and rapidly convergent to solutions in a large conformational search space of RNA structures. It borrows from the processes of biological evolution using operations such as mutation, recombination, and reproduction and a selection criteria based on the idea of the survival of the fittest. The algorithm has been designed to run on the MasPar MP-2 massively parallel computer. Because of the highly parallel nature of the algorithm it lends itself well to the MasPar architecture. It computes 16,384 RNA conformations at each generation. It utilizes a random but structured information exchange at each generation that allows the algorithm to iterate towards an optimal solution. The rate of convergence is basically exponential as a function of generation number. At the present time, the algorithm appears to be robust, converging to the optimal or almost optimal structures with several sequences. As a matter of fact, the genetic algorithm uncovered some unknown bugs that existed in a commonly used dynamic programming algorithm by finding structures that had better free energy values than what the dynamic programming algorithm was producing. This algorithm is proving to be very useful from several points of view; 1) it is pointing towards potentially interesting characteristics of folding patterns in RNA; 2) it is pointing the way for the eventual inclusion of mechanisms for RNA pseudoknot prediction; 3) it is exploring the capabilities of genetic algorithms to this type of problem and computer architecture which in turn will allow this algorithmic methodology to be applied to other domains such as protein folding; and 4) it is providing valuable insights and understandings of parallel algorithms which are becoming more essential for grand challenge types of problems.

As part of the massively parallel computational effort on the MasPar we have obtained BLAZE, a massively parallel version of the Smith-Waterman sequence comparison algorithm which is capable of performing amino acid comparisons at the rate of 220,000,000 residues per second with 16,384 processors. This

comparison includes affine gap penalty calculations which improves sensitivity. Other high speed sequence comparison algorithms available do not include gap penalty calculations and most are considerably slower. In addition, sequence alignments may be produced as well as the ability to vary FAM matrices and gap penalty parameters.

An example of the improvement in sensitivity that BLAZE is capable of producing involved a bacteriophage T4 segA protein which it turns out has an interesting but non-obvious functional similarity with an *N. crassa* mitochondrial apocytochrome b protein. If one were to perform a similarity search using the default settings of BLAZE, BLAST and FASTA, only BLAZE would immediately reveal the important similarity. As a matter of fact, BLAZE reports the result in the seventh position in the output hit list while FASTA produces it at position 87 and BLAST at position 58 (Gary Smythers and Mark Gunnell).

We also have ported to the MP-2 the Zuker suboptimal RNA folding code which is capable of folding large RNA sequences that are about 9000 nucleotides in length without any special packing. This is larger than the CRAY YMP is capable of folding. HIV is an example of such a sequence. It has also been demonstrated that the MP-2 can fold such sequences faster than a single processor of the CRAY YMP thus giving a considerable price performance advantage. It is also believed that further performance enhancements are achievable (Jih-Hsiang Chen, Joe Navetta, Jacob Maizel).

We have also recently received software from the University of North Carolina for visualizing molecular docking. This program does molecular force calculations on the MasPar while interactively displaying the results of these calculations on a Silicon Graphics workstation. One may interactively manipulate a drug within the environment of a protein searching for the optimal placement of the drug. This may prove to be useful for computer modeling for drug design. A program such as this should show substantial improvement of performance given the improved floating point speed of the MP-2 (Joe Navetta and Jacob Maizel).

Work on the nucleic acid structure analysis system has continued in a variety of collaborations and directions. For example, the MasPar with the currently running versions of the genetic algorithm for RNA structure prediction and the fast suboptimal dynamic programming algorithm has been incorporated into this heterogeneous computing environment. This system has been developed for the analysis of RNA secondary and tertiary structure and runs on a SUN workstation. 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 and tertiary 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 and tertiary structure problem domain involving various software/hardware complexes available at the Frederick Cancer Research and Development Center and elsewhere. The system currently has a large number of functions that permit the use of algorithms that reside on



different nodes within and external to the FCRDC network. This includes the SUN'S, SILICON GRAPHICS, CONVEXes, VAXes, CRAY and the MasPar. These algorithms are invoked from the SUN 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 structures and substructures to determine which are similar and which are not. 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. One may activate a "significance" algorithm and display its results to determine regions of RNA that have potentially interesting structures. The ability exists to graphically display structures that are generated. One may then interact with the display to get 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 enhanced to include more functionality (Wojciech Kasprzak) thus allowing broader access to the research community. The SUN version of the system, which is currently being utilized by selected individuals, utilizes X-windows which permits the running of the system from many different types of workstations across networks, i.e., one can start up the system in the United States and interact with a display in Europe. The system has been used from various SUN workstations as well as Silicon Graphics workstations. Examples of the system use follow:

A problem involving AIDS related research that made extensive use of certain features of the RNA structure analysis system is described at the end of this report.

Another project involving the use of the RNA structure analysis system has involved research into tissue specific expression of human cytochrome P4502B7 (CYP2B7) mRNA which has been characterized (M. Czerwinski, T.L. McLemore, H.V. Gelboin and F.J. Gonzalez: Quantification of CYP2B7, CYP4B1 and CYPOR mRNAs in normal human lung and lung tumors; submitted to Cancer Research). The CYP2B7 mRNA contains internal "in frame" UGA codons of unique dual function: termination of translation or insertion of selenocysteine. The mRNAs of known selenocysteine-containing proteins share the selenocysteine insertion sequence (SECIS) motif and demonstrate a predictable folding into a somewhat unique stem-loop structure located at their 3' untranslated region. We searched for the SECIS structural motif in CYP2B7 mRNA (2907 nucleotides). This included the use of a unique structural pattern matcher in the RNA structure analysis system. A loose structural pattern was specified based upon the published structural motif and this was run against a database of 500 optimal and suboptimal RNA secondary structures. We looked carefully at the places where matches were found and located an area in the 3' untranslated region that had similar characteristics to that of the published selenocysteine structures. In addition, folding of just the 250 nucleotide subsequence centered around the area of interest showed the continued persistence of an unusually long stem loop structure. Also, we examined the sequence and structural characteristics

of CYP2B6 (expressed in liver without selenocysteine). Comparing CYP2B7 with CYP2B6 shows that the two sequences are highly homologous. However, CYP2B7 has a 12 nucleotide deletion in the 3' untranslated region and expresses selenocysteine. The main structural difference between the two sequences in the area of interest is a Y shaped structure in CYP2B6 as opposed to the linear structure in CYP2B7. This effort provides supporting evidence for insertion of selenocysteine CYP2B7 enzyme and may help to elucidate the mechanism of cotranslational incorporation of this rare amino acid (Maciej Czerwinski and Wojciech Kasprzak).

Collaborative efforts (Kathleen Currey) have continued in looking at the 5' non-coding region of Polio-virus RNA. We are examining the effects of single random mutations and the likelihood of whether they will result in local or global rearrangements in structure. Using structural comparison techniques, provided for in the RNA structure analysis system, we are able to group the structural rearrangements. We have observed global rearrangements, local rearrangements, and no changes in structure. When the local environment of each mutation is carefully examined, one notes that mutation sites tend to preserve their strandedness or base pairedness 60% of the time. When, preserved, the single stranded regions frequently maintain their local environment (i.e. bulge loop, internal loop, etc.) though occasionally may switch to another structure. Preserved double stranded regions may preserve their stem and local structure but as frequently may form a different stem. The local surrounding structure may or may not be preserved. Less frequently, a global rearrangement also occurs. Substructures have been noted that are preserved despite multiple mutations within that substructure, e.g., a hairpin consisting of two stems separated by a bulge loop had eight mutations in the stems yet the basic hairpin structure was preserved despite individual base pairings being disrupted. It has been shown that single base changes can determine the switch from neurovirulence to non-neurovirulence and this has been related to structure (Skinner, et. al.). We are looking at these specific sites as well as others where we have data showing that mutations (insertions and deletions) affect function (Trono, et. al.). Our purpose is to examine and characterize structure/function relationships using our mutational data as a guide.

A new method for discovering motifs in protein sequences has been developed and is undergoing testing (submitted to NAR). The method involves an automatic two step process: (1) find (approximately) common motifs among a small sample of sequences; and (2) test whether these candidate motifs are present in all sequences. To accomplish the steps, the algorithm first locates some 'basic' motifs in the sample using existing string matching algorithms. It then refines the results by 'gluing' the basic motifs to form longer ones. The algorithm makes use of a previously developed algorithm for approximate string matching with variable length "don't cares" to detect the occurrence frequency of motifs. Experimental results obtained by running these algorithms on three families of functionally related proteins demonstrate good performance of the method (Dennis Shasha).

Also, a systolic algorithm was implemented on SPLASH which not only computes edit distances but also includes alignment information. Because of the extensive pipelining in the systolic array, computing an alignment on SPLASH takes the same amount of time as computing the edit distance. Compared to conventional computers, SPLASH performs several orders of magnitude faster. At the current time a new systolic array configuration, SPLASH II, is being developed at the Super Computing Research Center which should substantially enhance SPLASH's performance both in its computational and I/O capabilities. Eventually the sequence comparison algorithm will be moved to SPLASH II. At the current time the comparison algorithm is running on a SPLASH II emulator. The actual porting to the hardware should occur shortly. This approach may eventually lead to a relatively inexpensive mechanism for doing rapid extensive sequence comparisons with a plug in board to a standard workstation (Mort Schultz).

#### AIDS Related Research:

The nucleocapsid protein NcP7 of HIV-1 is important for encapsidation of the virus genome, RNA dimerization and primer tRNA annealing *in vitro*. From a combination of gel shift experiments and computational RNA structure analysis it has been shown that NcP7 binds specifically to an RNA sequence (see ref.). In addition, mutational analysis of the RNA shows that the predicted stem and loop structure plays a critical role. Results show that NcP7 binds to a unique RNA structure within the Psi region. Also, this structure is necessary for RNA dimerization. It is proposed that NcP7 binds to the RNA via a direct interaction of one zinc binding motif to a stem loop structure in one RNA molecule followed by binding of the other zinc binding motif to a motif in the other RNA molecule (Eric Baldwin).

The determination of the RNA structure discussed above used several features within the RNA structure analysis system. It is further proof of the concept that this system can be used successfully with collaborative experimentation. Specifically, one of the studies involved mutating the linker region between the two stem loop structures so that Gel shift experiments could be performed to determine how the mutations affected nucleocapsid binding. However, the determination of exactly what mutations should be applied to the linker while preserving the overall structure is not a simple matter, since for example, single base mutations caused changes in the overall structure. To determine the appropriate mutations required we used the mutational feature of the RNA structure analysis system with the morphology clustering algorithm (previously reported). Several randomly mutated sequences were generated and clustered with the wild type. Interestingly, only two of these mutated sequences folded optimally into the wild type form indicating the difficulty one would have if this procedure were tried manually. One of these mutated sequences was used for further gel experimentation. Results indicated that the linker region sequence was not significant for dimer formation.

In another part of the verification process for the dimer structure, 400 structures were compared which were generated from eight different HIV-1 sequences drawn from GENBANK. All of these sequences were about 322 nucleotides long and were centered on the Psi region. The 400 structures were compared in the Psi region again using the morphology comparison algorithm. The overwhelming majority of the predicted structure (224) produced a pair of stems and loops separated by a single strand linker. Most of the remaining structures maintained stem loop1, and stem loop2. Again, this gave very good evidence concerning the validity of the structure. This would have been a very difficult task to do manually.

**Publications:**

Sakaguchi K, Zambrano N, Baldwin ET, Shapiro BA, Erickson JW, Omichinski JG, Clore GM, Gronenborn AM, Appella E. Identification of a binding site for the human immunodeficiency virus type 1 nucleocapsid protein. PNAS, in press.

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

PROJECT NUMBER

201 CB 08387-06 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Cellular Nanoanatomy and Topobiology

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Section LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Victor Romanov Visiting Fellow LMMB, NCI  
 Hector Caruncho, Ph.D. Guest Researcher LMMB, NCI  
 Christina Risco, Ph.D. Postdoctoral Fellow PRI/FCRDC  
 Shen Rulong, M.D. Postdoctoral Fellow ABL/FCRDC  
 Eliana Munoz, M.T. Research Associate PRI/FCRDC  
 José Carlos Mirones, M.D. Guest Res. (Ministry of Defense. Port.) PRI/FCRDC

COOPERATING UNITS (F ANY)

I. Tsarfaty, D.L. Faletto, G.F. Vande Woude, P. Sutrave, S. Rong, S.H. Hughes, J. Resau, ABL/FCRDC; R. Anadon, Dept. Fundamental Biology, Univ. Santiago de Compostela, Spain; (continued)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

TOTAL STAFF YEARS:

6.0

PROFESSIONAL:

6.0

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.)

For the past year, our researchers have established a network of collaborative studies that link our expertise in immunogold labeling cytochemistry and in a unique system of methods developed in our laboratory over the past decade (fracture-label, label-fracture, fracture-flip, and simulcast). To this end, postdoctoral fellows bring their backgrounds and research interests and actively seek projects where answers depend on cytochemical and ultrastructural analysis. Seven main projects are described: (A) immunogold cytochemical localization of oncogenes and oncogene-related proteins (Rulong); (B) immunocytochemical study of the retroviral infection (Risco); (C) effects of the interaction of bacterial endotoxins on the plasma membrane of macrophages and pneumocytes (Risco); (D) nanoanatomy and topochemistry of the cell surfaces of protozoan parasites (Pimenta); (E) ultrastructural aspects of 67 kD laminin receptor and its precursor processing in metastatic potent cells (Romanov); (F) freeze-fracture immunocytochemical study of the expression of native and recombinant GABAA receptors (Caruncho); and (G) ultrastructure and response to darkness of goldfish meninges (Caruncho). The emphasis of our research reflects an attempt where a diverse group of scientists, all with a strong background in cellular ultrastructure and topochemistry, provides unique, critical skills and approaches to scientists in areas as diverse as oncogene biology, molecular neurobiology, virology, bacteriology, parasitology, as well as the biology of metastatic cells. Within our Section these scientists, while pursuing well-focused projects, interact mutually and develop the basis for future independent research careers.

## Cooperating Units (Continued.):

G. Puia, E. Slobodyansky, E. Costa, Fidia-Georgetown Institute for the Neurosciences, Univ. Georgetown, Washington, DC; C. Romero, M. Asunción Bosch, Dept. Biochemistry & Molecular Biology I, Universidad Complutense de Madrid, Spain; L. Menéndez-Arias, S. Oroszlan, Lab. Molecular Virology & Carcinogenesis, ABL/FCRDC; P.F.P. Pimenta, Lab. Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, NIH; D. Mirelman, The Weizman Institute of Science, Rehovot, Israel; V. Castronovo, M. Sobel, Lab. Pathology, National Cancer Institute, NIH; D. Mirelman, The Weizman Institute of Science, Rehovot, Israel; E. Mialhe, Univ. Blaise Pascal, Paris, France; M. Pereira, Tufts Univ., Boston; K. Fukasawa, I. Givol, ABL/FCRDC.

## PROJECT DESCRIPTION

Major Findings:

A. Immunogold cytochemical localization of oncogenes and oncogene-related proteins (Rulong, Fukasawa, Rong, Tsarfarty, Faletto, Givol, Hughes, Vande Woude, Pinto da Silva).

1. Met. The Met protooncogene product (Met) was first discovered in a osteosarcoma cell line (HOS) treated with chemical carcinogen N-methyl-N'-nitro-N-nitrosoguidine (Cooper CS, Nature 1984;311:29-33). Its gene was amplified and overexpressed in spontaneous transformants of NIH 3T3 cells and in human gastric carcinoma cell lines. Recently, the Met protooncogene product has been shown to belong to the tyrosine kinase growth factor family, and hepatocyte growth factor/ scatter factor (HGF/SF) has been identified as its ligand. Our previous work had shown the Met protooncogene product and HGF/SF may be involved in the lumen formation in vivo and in vitro. In the tumorigenicity test in NIH 3T3 cells and in nude mice, human Met alone showed poor tumorigenicity; however, high tumorigenicity was observed when human Met was co-expressed with HGF/SF (Rong et al., Mol Cell Biol 1992;12:5152-8). When observing the histological slides of the tumor tissues induced by co-expression human Met and HGF/SF in nude mice, we found increased amount of microlumen. We deparaffinized these tumor tissues and reembedded them in Epon 812 for electron microscopy observation. By carefully checking semithin sections, we found carcinoma-like regions within the sarcoma background. Electron microscopic observation of these carcinoma-like areas revealed desmosome-like junctions normally seen only in carcinomas. Moreover, the primary tumor and their explanted cell cultures showed positive cytokeratin staining. This transformation is similar to the embryonic conversion of mesenchymal into epithelial cells that occur in kidney, testis, and ovary tissues during organogenesis. We show that kidney cells undergoing conversion from mesenchymal to epithelial cells express high levels of Met as well as vimentin and cytokeratins. These results imply that Met and HGF/SF may participate in the transformation process from mesenchymal cells into epithelial cells.

2. **Mos.** The mos oncogene was originally discovered as part of the retrovirus Moloney murine sarcoma virus, a virus that causes fibrosarcomas in mice, rats, and hamsters. In 1980, the cellular mos protooncogene (c-mos) was isolated from the mouse genome and was shown to have transforming activity (Vande Woude, 1992). Previous studies showed that the mos protein is important for seemingly contradictory processes: the initiation and the arrest of meiosis. However, both the activation of mitosis promote factor (MPF) (the entry of oocytes into meiosis) and the stabilization of MPF (the arrest of oocytes in meiosis) indicate and there is an interaction between mos protein and the cell cycle regulatory proteins that compose MPF. These observations suggest that the mos protein is a stage-specific regulator of M phase (in meiosis or mitosis).

When mos is overexpressed in Swiss 3T3 fibroblasts by infection with v-mos containing Moloney sarcoma virus (Mo-MSV), a large number of cells detach from the culture dishes as "floaters" and growth is arrested at either the 2C or 4C phases of the cell cycle. These cells display several M-phase phenotypes including chromosomal condensation, the presence of proteins with epitopes recognized by phosphorylation-dependent M-phase specific antibodies, and high MAP kinase activity. The floaters eventually die over a period of 1-2 weeks. Examination of the floaters during this period revealed characteristics of cells undergoing apoptosis (a programmed cell death): i.e., nuclear fragmentation, specific degradation of DNA, EM observation found that the floaters are a uniform cell populations, with about 60-70% single nucleated, 30-40% binucleated, and less than 4-6% apparently dead cells. The single and binucleated floaters all have marked convolution of nuclear and cellular surface as well as condensed and segregated chromatin abutting the nuclear envelope and inside the nuclear matrix. Furthermore, the nucleoli lose their normal structural details, appearing as diffused ground masses uniformly electron dense clumps, some of them swelling to the size of 1/3 or half of the nucleus. The cytoplasm is condensed and crowded with numerous mitochondria and phagosomes. The cells show also an increased presence of rough endoplasmic reticulum and golgi complexes. This suggests that these cells are in the active metabolic and energy consuming stage (apoptosis is an energy-dependent process). Both nuclear and plasma membranes are intact and the organelles are well preserved. In contrast to the floaters, the mos transformed cells that remain attached to the culture dishes showed some early ultrastructural changes of apoptosis, while control swiss 3T3 cells were normal. These results suggest that the growth arrested M-phase phenotypes in somatic cells induced by mos overexpression leads to programmed cell death rather than necrosis (Rulong, Fukasawa, Pinto da Silva, Vande Woude, abstract sent to oncogene meeting, 1993).

3. **MUC1.** Characterization of the first human mucin gene (designated MUC1) resulted from studies of high molecular weight ( $M_r > 400$  kDa) glycoproteins (previously labeled as PUM, PEM, MAM-6, PAS-0, EMA, NPG, and DF-3) that occur in human breast milk and are highly expressed in breast and other adenocarcinomas (Hareveni et al., 1990; Ho Samuel et al., 1993). MUC1 is a complex glycoprotein characterized by high molecular weight, an amino-acid backbone rich in proline, serine, threonine and alanine residues, and a high content of hydrocarbons (mostly O-linked) comprising more than 50% of the mature glycoprotein (Hilkens et al., 1988).

One of the mAb (H23) recognizing a similar mucin molecule was obtained by us using a protein secreted by the breast cancer cell line T47D as immunogen (Tsarfaty et al., 1988). The protein recognized by the mAb was designated H23Ag (Keydar et al., 1989). Immunoperoxidase staining with H23 detected the H23Ag in the cytoplasm of 91% of all malignant breast tumor analyzed, whereas little or no staining was observed in adjacent breast tissue and normal breast tissue (Keydar et al., 1989). Elevated amount of this protein was also detected in sera of metastatic breast cancer patients, providing a new diagnostic tool to the breast cancer. Recently, the full length cDNA sequence of the MUC1 gene has been determined (Hareuveni et al., 1990). Part of MUC1 is composed of an array of 60 bp tandem repeat units which is highly conserved. Moreover, it has been shown recently, that most of the mAbs reacting with MUC1 recognize an epitope that includes the tetrapeptide proline, aspartic acid, threonine and arginine (PDTR) which constitutes part of the repeat unit. We used H23 to study its subcellular localization and its possible involvement in the interaction between cells.

NIH 3T3 cells were transfected with the transmembrane form of H23Ag. Transfected NIH 3T3 cells were tested for expression of the H23Ag with mAb H23. T47D breast cancer cell line was purchased from ATCC. LR Gold (The London Resin Co Ltd PO Box 29, Working Surrey England) low temperature post-embedding immunogold labeling combined with replica-labeling freeze-fracture and fracture-flip were used in these experiments. In the T47D cells, our results showed the colloid gold spheres were exclusively membrane associated. Intracellularly, the colloid gold spheres were associated with the membranes of secretory vesicles. On the cell surface, the gold particles were on the plasma membrane and the surface of microvilli. In some T47D cells, we could observe clear polarized distribution of the colloid gold spheres over the apical domain of the plasma membrane. Labeling was also intense in the intercellular space, where the concentration of the colloid gold spheres was proportional to the width to the intercellular space. This indicated that H23Ag may be related to the metastasis capability of the breast cancer. In NIH 3T3 cells transfected with MUC1 gene, cells that had been successfully transfected showed gold spheres associated to the plasma membrane and microvilli. Labeling over areas was much less than those of the T47D cells: in addition to membrane-associated labeling, there was some gold labeling of secretory vesicles. However, in cells that were not expressing MUC1, no label was seen. In T47D breast cancer cells, there was a large number of MUC1 proteins inside the cytoplasm that associated to the membranes of secretory vesicles, migrating to the membranes and the microvilli of microcolumn that formed within the cell during the cell culture. A label-fracture and replica staining of fracture-flip study of the MUC1 showed clear patched labeling of plasma membranes and microvilli consistent with the thin section results. However, label-fracture showed that the gold spheres are preferentially partitioned to the protoplasmic face of the plasma membrane and some of the microvilli labeling revealed a spiral labeling pattern that may explain the patched distribution pattern in thin section samples.



4. **Bcl-2.** The bcl-2 protooncogene was a new oncogene found in a variety of B-lymphoid tumors. Functional studies of bcl-2 proved it inhibits apoptosis (programmed cell death) (Korsmeyer, *J Immunol Today* 1992;13:285-8). Currently, the genes and mechanisms that control programmed cell death are the object of intense interest in oncogene research. We have performed postembedding immunogold labeling electron microscopy to study chicken embryo fibroblasts (CEF) that overexpress the human bcl-2 $\alpha$  protein protooncogene. To this end, we infected CEF with a replication competent RCASBP retroviral vector. Our immunogold labeling showed for the first time at the ultrastructural level, the clear association of bcl-2 $\alpha$  protooncogene protein with the nuclear envelope and the rough endoplasmic reticulum membrane.

**B. Immunocytochemical study of the retroviral infection** (Risco, Menéndez-Arias, Pinto da Silva, Oroszlan).

Since the discovery of HIV and HTLV as the etiological agents causing AIDS and T-cell leukemia in humans, respectively, extensive studies on their replication and life cycle have been carried out. However, our knowledge on the early events of virus infection is limited. It is assumed that the onset of reverse transcription (RT) takes place in an organized structure within the cytoplasm (probably in "intact" capsids). The viral and cellular factors triggering the initiation of the RT reaction that lead to the formation of DNA-containing infective entities are not known. Determination of the molecular events involved would probably facilitate the development of potential antiviral agents other than RT inhibitors. To achieve this goal, we are applying immunogold methods to study the entry and fate of viral components after virus infection. These methods have been successfully applied by our group to the study of proteins associated to immature mouse mammary tumor virus (MMTV) (Menéndez-Arias et al., *J Virol* 1992;66:5615). Since the resolution and specificity obtained in this study were very good, we are now applying these techniques in the study of the early phase of the retroviral infection, using the murine leukemia virus (MuLV) and following the time course of the virus entry and the subcellular distribution of viral proteins. This study is providing new data about the elements involved in the first steps of the infection and the definition of the proteins that constitute the "integration complex" of the virus, with a resolution that cannot be obtained by cellular fractionation. After completion of the analysis of MuLV infection, we plan to extend our study to HIV.

**C. Effects of the interaction of bacterial endotoxins on the plasma membrane of macrophages and pneumocytes** (Risco, Romero, Bosch, Pinto da Silva).

Immunocytochemical methods have proved to be useful in the study of the interaction pathogens--target cells, as they provide basic information to define possible key interactions, usually in the context of rather complex processes (Risco et al., review in preparation). Our studies on the visualization of the interaction of bacterial endotoxins with macrophages (Risco, Pinto da Silva, *J Histochem Cytochem* 1993;41:601), have been extended to another target cell of the endotoxin action: the type II pneumocyte. The activity of these cells is essential for the maintenance of the lung function, as they produce and secrete

the pulmonary surfactant. This lipoproteic material has tensoactive properties that avoid alveolar collapse during respiratory movements. In endotoxemic processes, the metabolism of the type II cells and the integrity of the surfactant system are seriously affected, linked to respiratory failure, as is common in these pathologies. Using different EM techniques, such as thin-section, freeze-fracture, and fracture-flip gold labeling, we studied the intracellular membranous systems of the type II pneumocyte and their interrelations. This provided new data on the ontogeny of the surfactant storage bodies and the mechanisms of surfactant secretion (Risco et al., Lab Invest, In Press). The effects of the binding of a bacterial endotoxin on the microanatomy of the plasma membrane of the pneumocyte were also analyzed. High-resolution images of the pneumocyte surface showed that the insertion of the endotoxin of *Escherichia coli* in the pneumocyte plasma membrane creates lipidic "smooth" areas (devoid of particles) at the membrane surface. This might reflect local changes of membrane microviscosity, possibly the formation of fluid domains leading to functional alteration in particular proteins.

#### D. Nanoanatomy and topochemistry of the cell surfaces of protozoan parasites (Pimenta, Mirelman, Mialhe, Pinto da Silva).

1. *Entamoeba histolytica*. Human infections with *Entamoeba histolytica* are prevalent, particularly in the developing nations of the world, with an estimated 500 million cases per year. An increasing body of biochemical and molecular evidence indicates that there is most likely two genotypically different but morphologically similar organisms: (a) the pathogenic, isolated from sick individuals; and (b) the nonpathogenic, isolated from healthy carriers. In our study, we examined and compared thin section, freeze-fracture and fracture-flip images of well characterized pathogenic and nonpathogenic amebas. Observation of the cell surface of the parasite with fracture-flip reveals the presence of higher number of rounded particles on the cell surface of nonpathogenic trophozoites. Freeze-fracture shows the presence of linear arrays associated with pinocytosis area only on the cell surface of pathogenic trophozoites. Observation of thin sectioned parasites shows that the endocytic vacuoles in pathogenic and nonpathogenic *E. histolytica* present distinct localization of bacteria. All nonpathogenic trophozoites present a single bacteria inside the endocytic vacuoles. In contrast, all endocytic vacuoles appear to contain three to eight bacteria.

2. *Bonamia ostreae*. *Bonamia ostreae* is an Asctospora protozoa that parasitizes bivalve mollusks (*Ostrea edulis*). We are investigating the effect of magainins, peptide antibiotics secreted by the frog *Xenopus laevis*. This antibiotic is an amphipatic peptide that interacts with cell membranes, forming an anion channel. Our preliminary results (thin sections) showed that this antibiotic is able to penetrate the hemocytes (host cells) and affect the parasite viability, with no toxic effect over the host. Initially, treated parasites secreted their "dense bodies" (lysosome-like organelles) and underwent autolysis. Freeze-fracture and fracture-flip are being used to understand the interaction of the parasite with host cells, as well as the effect of the magainin.

3. **Trypanosoma cruzi.** *Trypanosoma cruzi* invades mammalian cells and provokes a serious tropical disease in several undeveloped countries. Binding of the parasite and its penetration of target cells was recently described as mediated by a surface protein named "penetrin." The recombinant protein localized on the surface of *Escherichia coli* induces the bacteria that express it to adhere to and penetrate nonphagocytic cells. We are using thin-section and immunogold labeling to compare details of the penetration of recombinant bacteria and parasites. Preliminary observations of cells infected with recombinant and arrested at different times, showed that, after penetration of the host cells, the bacteria escape from the vacuoles and are free in the cytoplasm. We intend to label thin-sections to observe the possible involvement of penetrin in the mechanism of vacuolar escape of the parasite. We also intend to use freeze-fracture and fracture-flip to examine this protein over the parasite cell surface.

E. **Ultrastructural aspects of 67 kD laminin receptor and its precursor processing in metastatic potent cells** (Romanov, Castronovo, Sobel, Pinto da Silva).

The 67 kD laminin receptor (67LR) is responsible, at least in part, for the high affinity of cancer cells for laminin, a major glycoprotein in basement membrane. A 37 kD polypeptide (37LRP) has been identified as the precursor of 67LR. Although 37LRP does not have signal peptide or classical features of an integral membrane protein, there are strong evidences that it is a membrane-associated molecule. 37LRP is abundantly present in the cytoplasm, and a protein identical to 37LRP has been described as a protein associated to cytoplasmic polysomes, suggesting a multifunctional role for this molecule. In order to better understand the cellular localization of the 67LR and its precursor, transmission electron microscopic studies of human melanoma A2058 cells were carried out using the immunogold technique and a variety of antibodies, including affinity-purified antibodies directed against 37LRP cDNA-derived synthetic peptides and monoclonal antibodies MuLC5 and MuLC6, raised against human small cell lung carcinoma N592 microsomal cell fraction. All the anti-67LR and anti-37LRP antibodies were found to bind specifically to indistinct cytoplasmic structures. A reactivity with the cell surface was also clearly observed but was not dramatic. Immunogold double-labeling revealed that monoclonal antibodies raised against mature 67LR and antibodies directed against the 37LRP co-localized in these structures. After addition of soluble EHS laminin to A2058 cells in suspension, these structures moved in a few minutes toward the plasma membrane with an increase of the number of receptors at the surface of the cell. The number of 67LR positive structures in the cytoplasm also increased. This stimulation effect was specific for laminin and was not induced by fibronectin or albumin. These data indicate that 67LR is cell surface associated but in the resting cells most of the receptors are localized in indistinct cytoplasmic structures that are exported toward the cell surface upon stimulation with laminin. This process, which has not been reported previously, could be determinant in the interactions between the cells and laminin, since it provides more binding sites for the ligand, during tumor invasion and metastasis.

**F. Freeze-fracture immunocytochemical study of the expression of native and recombinant GABA<sub>A</sub> receptors (Caruncho, Puia, Slobodyansky, Pinto da Silva, Costa).**

To assess the density and distribution of native and recombinant GABA<sub>A</sub> receptors, we used label-fracture and fracture-flip technologies combined with immunocytochemistry using monoclonal and polyclonal Abs directed against the extracellular domain of the GABA<sub>A</sub> receptor protein located in the freeze-fracture replicas. In cortical neurons there is a high density of GABA<sub>A</sub> receptors on both soma and dendrites with some areas where the density of receptors is higher, but there are no well-defined clusters. In cerebellar granule cells, most of the receptors are distributed in round clusters both in neurites and soma. In astroglial cells, the receptor density is lower than in neurons and only occasionally they appear in clusters. In cells transfected with cDNAs encoding for various molecular forms of GABA<sub>A</sub> receptor subunits, the receptor density is moderate when cDNAs for  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits are cotransfected; however, on cells cotransfected with cDNAs for  $\beta$  and  $\gamma$  subunits, the receptor density is significantly lower. Recombinant receptors appear randomly distributed and occasionally they aggregate in small groups.

**G. Ultrastructure and response to darkness of goldfish meninges (Caruncho, Anadon, Pinto da Silva).**

**1. The morphology of teleost meningocytes as revealed by freeze-fracture.** We described the morphology of the cells in the three layers of the teleost endomeninx, as viewed by freeze-fracture. The cells of the outer endomeningeal layer are fusiform and closely packed, with interdigitations that hold the cells together, gap junctions and a few strands of particles resembling tight junctions, but no desmosomes. The intermediate layer is formed by a single layer of flattened and elongated cells with rectangular shape, and well developed junctional complexes (gap junctions, tight junctions, and desmosomes). These cells also show numerous plasmalemmal vesicles ( $6.5 \pm 1.3/\mu\text{g}^2$ ) in the upper (in contact with the outer layer) and lower (in contact with the inner layer) membranes. Cross fracture of these cells shows many membrane-bound and free vesicles. The inner layer is formed by spindle-shaped cells with wide intercellular spaces filled with a granular matrix and collagen fibers. The density of intramembrane particles is higher than that in the meningocytes of the outer and intermediate layers. The morphology of the teleost endomeninx appears similar to that of the leptomeninges of birds and mammals, but the outer endomeningeal layer of teleosts (which resembles the arachnoid of elasmobranchs and amphibians) appears different from any cell layer in the meninges of amniotic vertebrates.

**2. Alterations in the intermediate layer of goldfish meninges during adaptation to darkness.** The morphological changes in the intermediate endomeningeal layer of the goldfish brain during light and dark adaptation were studied by freeze-fracture. During the different steps of the adaptation, we found no significant changes in the density of intramembrane

particles or nuclear pores in these cells. The density of plasmalemmal vesicles in the meningocytes surface was increased in the groups maintained in the dark for 48 and 72 hours (maximum), and then decreased in the group maintained for 96 hours in the dark, to a value similar to that of the control group. We also found morphological changes in the junctional complexes: the number of tight junction strands and desmosomes in the lower membranes of meningocytes (in contact with the inner layer) was decreased in the group maintained in the dark for 24 hours, and then in the groups maintained in the dark for 48, 72, or 96 hours was similar to that in the control group. In the upper cell membranes (in contact with the outer layer) of meningocytes from the group maintained in the dark for 48 hours, we found an increase in the surface occupied by gap junctions. In addition, gap junctions were absent in the lateral membranes of meningocytes from animals maintained in the dark for 72 hours. The morphology of gap junctions in the group maintained in the dark for 96 hours was similar to that of the control group. These results suggest that the cells of the teleost intermediate endomeningeal layer suffer important changes in activity during adaptative experiments.

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Caruncho J, Pinto da Silva P. Alterations in the intermediate layer of goldfish meninges during adaptation to darkness. *J Submicrosc Cytol*, in press.

Caruncho H, Pinto da Silva P, Anadon R. The morphology of teleost meningocytes as revealed by freeze-fracture. *J Submicrosc Cytol*, in press.

Caruncho H, Puia G, Slobodyansky E, Pinto da Silva P, Costa E. Freeze-fracture immunochemical study of the expression of native and recombinant GABA receptors. *Brain Res*, in press.

Risco C, Romero C, Bosch MA, Pinto da Silva P. Rat type II pneumocytes revisited: intracellular membranous systems, surface characteristics, and lamellar body secretion. *Lab Invest*, in press.

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

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Function of the Transmembrane Domain of Glycosyltransferases

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Pradman K. Qasba, Ph.D.                      Research Chemist                      LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Arni Masibay, Ph.D.	Staff Fellow	LMMB, NCI
Elizabeth Boeggeman, Ph.D.	Senior Staff Fellow	LMMB, NCI
Petety Balaji, Ph.D.	Visiting Fellow	LMMB, 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 STAFF YEARS:

1.55

PROFESSIONAL:

1.55

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.)

Analyses of the cDNA sequences of glycosyltransferases have shown that these enzymes have inverted membrane topology that consists of a short amino-terminal cytoplasmic tail, a hydrophobic transmembrane anchor domain and the carboxyl-terminal catalytic domain. To examine the role of the transmembrane domain a series of mutants and chimeric cDNA of  $\beta$ -1,4-galactosyltransferase ( $\beta$ -1,4-GT) were constructed by PCR, transiently expressed in COS-7 cells, enzyme activities measured and the protein localized in the cells by subcellular fractionation or indirect immunofluorescence microscopy. Deletion analyses of the amino-terminal region show that the first 21 amino acids of  $\beta$ -1,4-GT are not essential for the stable production of the protein and are consistently localized in the Golgi apparatus. However, as reported earlier, the deletion of the transmembrane domain abolishes the stable expression of this protein in mammalian cells. In addition, analysis of hybrid constructs showed that residue 1-25 of  $\alpha$ -1,3-GT can functionally replace the  $\beta$ -1,4-GT amino-terminal cytoplasmic and transmembrane domain (residues 1-43). This fusion protein also showed Golgi localization. On the other hand, protein fused to the transmembrane domain of  $\alpha$ -2,6-sialyltransferase ( $\alpha$ -2,6-ST) needed additional COOH-terminal sequences flanking the domain for stability and Golgi localization. Substitution of Arg24, Leu25, Leu26 and His33 of  $\beta$ -1,4-GT transmembrane by Ile or substitution of Tyr by Ile at positions 40 and 41 coupled with the insertion of four Ile at position 43 released the mutant proteins from the Golgi and were detected on the cell surface. Our results show that a) the transmembrane domains of  $\beta$ -1,4-GT,  $\alpha$ -1,3-GT, and of  $\alpha$ -2,6-ST along with its stem region all play a role in Golgi targeting, and participate in a common mechanism that allows the protein to be processed properly and not be degraded in vivo; b) increasing the length of the transmembrane domain overrides the Golgi retention signal and directs the enzyme to the plasma membrane; and c) the length of the hydrophobic region of the transmembrane domain is an important parameter, but is not sufficient by itself for Golgi retention.

## PROJECT DESCRIPTION

Objective:

To identify the sequence and/or structural motif of the transmembrane region of glycosyltransferases and its function in the biogenesis of the enzyme

The protein sequences of glycosyltransferases, derived from the cDNA sequences, have implicated that these enzymes have inverted membrane topology that consists of short amino-terminal cytoplasmic tail, a hydrophobic transmembrane anchor domain and the carboxyl-terminal portion of the protein that carries the catalytic domain. The objective of the present studies is to determine by the recombinant DNA methodology, a) the sequence region of the trans-Golgi transferases that functions as the Golgi targeting sequence, and b) if there are common residues and/or a structural motif within this sequence that is being recognized during the targeting process.

Major Findings:**The Transmembrane Domain of  $\beta$ -1,4-GT is Necessary for Protein Production in COS-7 Cells**

To determine whether the cytoplasmic or the transmembrane domain is important for production of  $\beta$ -1,4-GT in COS-7 cells and to investigate what effect these domains have on Golgi localization of  $\beta$ -1,4-GT, a series of deletion mutants were constructed. Measurement of the enzyme activity and the Western-Blot analysis of the extracts of the cells transfected with the deletion mutants showed that the first 21 amino acid residues of the cytoplasmic region of  $\beta$ -1,4-GT are not essential for its production in COS-7 cells. The results show that the stable expression of this protein is sensitive to the deletions in the transmembrane region since removal of the first five amino acids of the transmembrane segment also abolishes the observed activity and stable protein levels compared to the wild-type protein. Deletion of the transmembrane region may result in the protein not entering the secretory pathway due to the lack of an intact signal anchor.

**The Bovine  $\alpha$ -1,3-GT Transmembrane Region can Replace Bovine  $\beta$ -1,4-GT while the  $\alpha$ -2,6-ST Transmembrane Domain Requires Carboxyl-terminal Flanking Sequence**

Since  $\beta$ -1,4-GT is but one member of a group of glycosyltransferases that exhibit conserved topological structures our hypothesis was that the corresponding transmembrane regions of other glycosyltransferases may directly substitute for the transmembrane segment of bovine  $\beta$ -1,4-GT. Assembled DNA segments coding for the cytoplasmic and transmembrane regions of  $\alpha$ -1,3-GT or



$\alpha$ -2,6-ST which would replace the corresponding transmembrane segment of  $\beta$ -1,4-GT were used for the generation of chimeric constructs to give rise to fusion proteins. The DNA segment coding for the cytoplasmic and transmembrane regions of  $\alpha$ -1,3-GT can replace the coding sequence for  $\beta$ -1,4-GT transmembrane domain. On the other hand  $\alpha$ -2,6-ST cytoplasmic and transmembrane domain (aa 1-29) substituting for the  $\beta$ -1,4-GT transmembrane domain, does not produce a stable protein. However, if the flanking sequences that code for a part of the neck or stalk region of  $\alpha$ -2,6-ST (aa 30-44) are added to the construct, a stable active protein is produced again.

#### Localization of Wild-Type, Deletion Mutants and Hybrid Proteins of Bovine $\beta$ -1,4-GT

$\beta$ -1,4-GT has traditionally been located within the Golgi compartment, but has also been detected on the plasma membrane of many cell types. Recently it has been suggested that the 402 residue long full-length protein and the short form of  $\beta$ -1,4-GT (residues 1-13 deleted) are targeted to different subcellular compartments. To better understand the mechanisms underlying the sorting of this glycosyltransferase, subcellular fractionation and immunofluorescence experiments were performed to see where the overexpressed proteins of wild type and deletion mutants of  $\beta$ -1,4-GT are targeted. Comparison of the subcellular fractionation profile of the pLbGTF with pLbGTD14 transfected cells show no significant difference, indicating that both full length and short form of  $\beta$ -1,4-GT are targeted mostly to the Golgi fraction. Furthermore the immunofluorescence data show that most of the over-expressed full length and short form of the protein are present in the Golgi and not on the cell surface. The localization of the fusion proteins produced from the hybrid constructs also showed that majority of the protein is directed to the Golgi compartment.

#### Effect of Mutations on Hydrophobicity of the Golgi-Retention-Signal Domain and on Cellular Localization of Bovine $\beta$ -1,4-GT

The amino acid sequence of  $\beta$ -1,4-GT transmembrane region has neither any homology with the transmembrane region of various glycosyltransferases cloned so far, nor with the transmembrane domains of integral membrane proteins. When the membrane-spanning region of  $\beta$ -1,4-GT was modeled into an  $\alpha$ -helix, using InsightII from Biosym Inc. as a molecular modeling package, we noticed that the polar residues line up on one side of the  $\alpha$ -helix. We began examining membrane-spanning domains of other proteins that were targeted to the cell surface, and observed that a majority of these proteins had stretches of hydrophobic residues uninterrupted by any charged residues on one side of the alpha helix. In contrast, proteins targeted to other compartments, such as, the Golgi or ER have this hydrophobic stretch interrupted by a charged or polar residue(s). To calculate the hydropathic index of each residue within the transmembrane domain of these various proteins we used the Kyte and Doolittle hydrophobic scale and a window of 7 amino acids. The first positive number of this stretch was then considered residue number 4 for comparative plotting purposes. The adjusted residue number vs the hydropathic index was plotted.

The length of the transmembrane was determined by counting the number of residues within this positive hydrophobic stretch. The length of the transmembrane domain based on the number of residues with positive hydrophobic index fall into two basic categories: one with a broad and the other with a short hydrophobic region. The proteins targeted to the plasma membrane tend to have a broader hydrophobic region, and the hydrophobicity values are greater compared to proteins targeted to the Golgi, suggesting a possible relationship between length of the transmembrane domain and their localization. Based on these analysis we investigated if by increasing the hydrophobic index of the signal-anchor domain of the Golgi-associated  $\beta$ -1,4-GT one could target the enzyme to the cell surface. These mutations were accomplished by substitution or a combination of substitution and insertion of Ile in the membrane-anchor domain. We determined the effect of these mutations on the localization of  $\beta$ -1,4-GT by fractionation of the cell extracts on sucrose gradients, whereby Golgi fraction is separated from plasma membranes, or by indirect immunofluorescence of transfected permeabilized and nonpermeabilized COS-7 cells. These results show that the Golgi retention signal can be disrupted by increasing the hydrophobic character of the transmembrane domain. Keeping intact the putative critical residues (Cys30 and His33) for Golgi retention and increasing the hydrophobic length of the membrane-spanning region, the mutant proteins were released from the Golgi and detected on the plasma membrane.

#### Publications:

Masibay AS, Balaji PV, Boeggeman E, Qasba PK. Mutational Analysis of the Golgi-Retention Signal of Bovine  $\beta$ 1-4galactosyltransferase. *J Biol Chem* 1993;268:9908-9916.

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

PROJECT NUMBER

Z01 CB 08396-05 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Information Theory in Molecular Biology

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Thomas D. Schneider, Ph.D. Senior Staff Fellow LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Paul N. Hengen	IRTA Fellow	LMMB, NCI
Stacy L. Bartram	SIP	LMMB, NCI
Maria Alavanja	SIP	LMMB, NCI
Jamie Fenimore	SIP	LMMB, NCI
Denise Rubens	Research Associate	PRI/FCRDC

COOPERATING UNITS (IF ANY)

Dhruba K. Chattoraj and Peter P. Papp, Laboratory of Biochemistry, NCI, Bethesda, MD; David Draper, Johns Hopkins, Baltimore, MD; Peter K. Rogan, Division of Genetics, University of PA, Hershey, PA; (continued)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

TOTAL STAFF YEARS:

3.50

PROFESSIONAL:

2.00

OTHER:

1.50

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.)

Shannon's measure of information is useful for characterizing the DNA and RNA patterns that define genetic control systems. It has been shown that binding sites on nucleic acids usually contain just about the amount of information needed for molecules to find the sites in the genome. This is a "working hypothesis" and exceptions can either destroy the hypothesis or reveal new phenomena. For this reason, we are actively studying several interesting anomalies. The first major anomaly was found at bacteriophage T7 promoters. These sequences conserve twice as much information as the polymerase requires to locate them. The most likely explanation is that a second protein binds to the DNA. We discovered that the F incD region has a three-fold excess conservation, which implies that three proteins bind there. We are investigating both anomalies experimentally. Thus the project has three major components: theory, computer analysis and genetic engineering experiments. The theoretical work can be divided into several levels. Level 0 is the study of genetic sequences bound by proteins or other macromolecules, briefly described above. The success of this theory suggested that other work of Shannon should also apply to molecular biology. Level 1 theory introduces the more general concept of the molecular machine, and the concept of a machine capacity equivalent to Shannon's channel capacity. In Level 2, the Second Law of Thermodynamics is connected to the capacity theorem, and the limits on the functioning of Maxwell's Demon become clear. Publications this year were mostly focused on Level 0, but work continues at all levels.

## Cooperating Units (continued):

Kenneth E. Rudd, National Library of Medicine, NIH, Bethesda, MD; Stanley Brown, ABL, Frederick, MD; George Pavlakis and Ralf Schneider, PRI, Frederick, MD; Steve Hughes and Steve Angeloni, PRI, Frederick, MD; Ed Brody, SUNY, Buffalo, NY; Sharlene R. Matten, Univ. of MD, College Park, MD; William S. A. Brusilow, Wayne St. Univ., Detroit, MI; John Spouge, National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD; Mark C. Shaner, Univ. of Maryland, College Park, MD; Ian Blair, Univ. of Pittsburgh, Pittsburgh, PA, R. Michael Stephens, MIT, Cambridge MA; Gaisela Storz, NIH, Bethesda, MD; Bill Benish, Harbor Beach, MI; Soren Brunak, Neil's Bohr Institute, Univ. of Copenhagen, Denmark and The Technical Univ. of Denmark; Martine Couturier, Universite Libre De Bruxelles, Belgium.

## PROJECT DESCRIPTION

Major Findings:

A series of projects are continuing that have a common thread of using concepts and language from the field of information theory to describe functional and structural sites in genes and proteins and to use experimental molecular biology to test hypotheses arising from sequence analysis. This approach proves to be broadly applicable to understanding many gene systems. Using these mathematical tools we recently discovered putative binding sites in DNA for the bacterial protein Factor for Inversion Stimulation (FIS) paired with sites known to be involved in DNA inversion. A week later the same tools revealed a pair of binding sites for the oxidation control repressor protein (OxyR). In both cases modern "footprinting" experiments were not sensitive enough to see the two adjacent sites. This kind of observation, obtained from sequence information, has happened repeatedly in our lab.

Splicing and Molecular Machines

Descriptions of biological molecules as machines using principles of information theory were presented at the Physics of Computation meeting in Dallas, Texas in October 1992. Soren Brunak of the Technical University of Denmark and the Neils Bohr Institute in Copenhagen, who uses neural networks to characterize splice junctions, expressed interest in our work and invited me to present our work to his institutes, during which we transported the sequence logo technology and applied it to several systems. Soren is finding it particularly useful for interpreting his neural networks.

A review of molecular machine theory for the journal Nanotechnology is still in revision (Schneider, 1993).

Work on the information analysis of splice junctions was accepted and published in J. Mol. Biol (Stephens & Schneider, 1992). The splice junction work has attracted considerable attention. Collaboration (George Pavlakis, Ralf

Schneider) continues using these techniques to predict effects of mutations on HIV splice junctions. With Steve Angeloni and Steve Hughes we are working to understand alternative splicing in chickens. Ed Brody is interested in extending our analysis to yeast. Our initial pass at analysis suggests that a different mechanism should be expected than that in humans.

### Individual Information

Work has begun on an important technical method, analysis of individual information. In this technique, individual binding sites are assigned an information content (measured in bits). This method has already been applied to FIS, OxyR, splice junctions, and ribosome binding sites among others and it can be applied to many genetic control systems. The distribution of individual information is approximately Gaussian. The zero point on the distribution is important since functional sites have positive information. By definition, the average of the distribution is Rsequence, which connects this method to all of our other work. Surprisingly, the consensus sequence lies far above the distribution and usually has a very small probability of being found in the natural population of binding sites. This method, along with the previously published sequence logo method, should displace most uses of the consensus sequences. A paper describing this method is in preparation.

### FIS Binding Sites

One of the important uses of individual information is as a theoretically based search tool which, unlike other tools reported in the literature, is not ad-hoc. Stacy Bartram has spent this year carefully collecting binding sequences of the FIS protein. This molecule bends DNA and is involved in many DNA rearrangement mechanisms. The individual information method was used to look at the promoter of the FIS gene itself. This promoter has 6 known FIS sites, but our analysis predicts that there may be as many as 20 sites. This leads to a working hypothesis for the action of FIS that assumes that when there is enough FIS protein in the cell, the promoter is obscured by a "cloud of bent DNA", effectively shutting off FIS synthesis. As FIS levels drop, the DNA would straighten out, allowing access to the promoter. In addition to this unique autoregulatory mechanism, it was found that DNA inversion regions have a pair of FIS sites situated next to each other. We do not yet understand the implications of this discovery for DNA rearrangement, but may test the prediction this summer by gel shift experiments to determine if the predicted FIS site can bind to the FIS protein.

### Two Fold Excess Information Content: T7 Promoter Project

The goal of this project is to determine by experimental methods the structure of bacteriophage T7 promoters, because the sequences at these promoters are more conserved than is necessary for the sites to be found. We continue to sequence the weak T7 promoters which we detect as smaller-than-usual ("tiny")

bacterial colonies. Preliminary results from 100 sequenced clones indicates that the tiny phenotype is more complex than originally thought. The tiny phenotype can be created by several changes across the entire promoter. This should allow us to characterize T7 promoters in more detail.

Custom software has been written to automatically handle the sequence data and we are now beginning to sequence 2000 mutated T7 promoters chosen at random. These will be used train a neural network to simulate the T7 RNA polymerase. This combination of bench work and computer analysis has never been attempted before. Our goal is to understand the three-dimensional structure of sequence contacts made by the T7 RNA polymerase.

The information in the sequences at wild-type T7 promoters is twice that expected from the number of sites and the size of the genome. The only hypothesis which has survived to explain this result is that a second protein binds at T7 promoters. Next we will be looking for the predicted second binding protein. If successful, this will confirm the information theory method for predicting binding proteins.

The sequence logo technique is giving us clues about what to expect and look for in this project. For many years I had imagined that I could take the sequence logo of the wild-type promoters and the sequence logo we obtain from the randomization experiment and then perform a "subtraction" to reveal the pattern to which the putative other protein binds. Recently I discovered that this is impossible. This demonstrates that bench work is essential for a full analysis of systems with excess information content.

### Three Fold Excess Information Content: F *incD* Repeat Project

Our discovery that the *incD* partition region of the F plasmid has three-fold excess information was published last summer (Herman and Schneider, 1992). Paul Hengen has obtained the *incD* region, confirmed its sequence and recloned it into a set of plasmids designed for detecting the three predicted proteins. Soon we hope to isolate the proteins and then to begin information theory dissection of the partition mechanism. This should reveal the DNA binding components required for the precise partitioning of newly replicated DNAs into daughter cells.

Hengen applied the Rs/Rf method to the replication sequences of pCU1 and discovered another case of three fold excess. The physical size of the pCU1 DNA repeats is different from that of *incD*, but the ratio is still close to 3.0, which demonstrates that the ratio itself is important.

### RepA Project

Information analysis of RepA binding sites, which are responsible for DNA replication of the P1 plasmid, showed an anomalous information peak in the sequence logo. In collaboration with Dhruba K. Chattoraj and Peter P. Papp we

synthesized many variations of the RepA binding site. We then selected, cloned, and sequenced 97 of those that still bind to RepA. The face of the DNA to which RepA binds was predicted by a new information theory method, and this was confirmed exactly by experimental footprinting. As predicted, the anomalous peak was absent from the experimental sequence logo but further work revealed that the situation is not so simple: the sites may be bound by a second protein, and they may contain a non-B DNA distortion. Our paper (in press) describes these results, and the information analysis of several other genetic control systems.

In collaboration with Martine Couturier, this project has been extended to analysis of other replication proteins. The evolutionary relationships of various plasmid replication regions are now being revealed by sequence logos.

#### **Eukaryotic Enhancer Project**

The yeast protein GCN4 binds to enhancer sites to stimulate transcription. Mark Shaner and Ian Blair analyzed the binding sites of GCN4 using information theory methods and discovered that current models for the binding sites are probably wrong. This prediction was confirmed by the recent publication of X-ray images of the protein. Shaner also discovered that GCN4 sites do not contain enough information for them to be found in the genome. His explanation is that the missing information is to be found in the TATA sites, and further information analysis supported this model. We have almost completed two papers describing these results.

#### ***E. coli* Database Project**

Kenn Rudd has collected all known *E. coli* DNA sequences as a continuous clean data set representing 50% of the genome. These data provide a wonderful platform for information theory analysis because the hard work of removing the numerous errors and duplications found in GenBank has been done. In collaboration with Rudd, we have analyzed over 1000 *E. coli* ribosome binding sites (Rudd and Schneider, 1992). This data set is nearly 10 fold larger than the original analysis done in about 1984, and thus it offers a refined look at the structure of the sites. A surprising but tentative result is that the information content of the binding sites is not sufficient for them to be located in the genome. Several explanations are possible, ranging from data problems to a spectacular mathematical proof that ribosomes scan the RNA linearly. We plan to investigate this new anomaly in detail and to analyze many other binding sites in *E. coli*.

### Other Binding Sites

A project to determine how RNA editing works was started (Bill Benish). The patterns recognized by the editing machinery have eluded others because they tried the consensus sequence method. The information theory methods quickly revealed an interesting pattern, but there are difficulties in interpretation because of the coding sequences.

In 1990, I analyzed the OxyR binding sites for Gaisela Storz. Recently she has created a set of synthetic binding sites and asked for help analyzing them. As mentioned above, we used the individual information technique to discover a likely case of a second OxyR binding site next to a known one. This is consistent with a change in the footprinting observed between oxidized and reduced conditions, but further experimental work will be required.

### A New Structure for GenBank

In the recent past GenBank placed such a large emphasis on catching up on sequence entry that other problems in data organization have accumulated. Errors, omissions, duplications and inconsistent sequences have led to this situation. I have submitted a paper describing and proposing a specification for an advanced sequence database which should solve these problems, and which would save much wasted effort world-wide.

### Sequence Logos and Molecular Phylogeny

As a replacement for consensus sequences, the sequence logo method for showing the patterns at binding sites continues to be used both by us and by other groups. Peter Rogan and I wrote a paper (Rogan et al., 1993) on a new method for using sequence logos to help phylogenetic studies. A sequence logo was created for the entire 28S rDNA sequence from several species, and this was used to help identify two regions of conservation surrounding a region of divergence. The conserved regions were used for PCR amplification of the divergent region. This method was successfully applied to species not included in the original analysis, so it promises to be useful for phylogenetic placement of an unknown DNA sample.

### Ribosomal RNA Project

In collaboration with David Draper at Johns Hopkins University, we are investigating the structure of ribosomal RNA binding sites for the ribosomal L11 and S4 proteins. The project pushes the limits of information theory analysis in two directions. First, we plan to analyze regions on the order of 500 bases, which are relatively large in comparison to previous studies. Second, because RNA has structures which correlate one position to another through base pairing, the simple 2-dimensional sequence logo must be extended



into 3-dimensions. This project should reveal the secondary structure of the rRNAs. Presumably this will be the same structure that many other groups have been determining by other methods; however, the new method may reveal features previously overlooked. Indeed, we have recently obtained some sequences with a large number of mutations, and many are compensatory in a way consistent with the known structures.

#### DNA Sequencing Project

A potentially patentable method concerning DNA sequencing is being investigated.

#### Synthetic Enzymes Project

Stanley Brown and I are collaborating on a method for creating a functional enzyme de novo. This project may also lead to a patent.

#### Unc Operon Perceptron Analysis.

I analyzed the E. coli unc operon using a neural network technique invented by Gary Stormo and me in 1982. Unexpectedly, I detected a ribosome binding site in the middle of the uncB gene. Sharlene R. Matten and William S. A. Brusilow recognized that this could explain why translation seems to decrease in the middle of uncB, a feature that had been puzzling them. They have now shown experimentally that downstream translation is increased if the putative stall sequence is disrupted. This work is in preparation as a paper.

#### Multiple Sequence Alignment

Michael Stephens is continuing to develop an information theory based multiple alignment program.

#### International Electronic News Group

I continue to guide and support the bionet.info-theory news group in which we discuss the use of information theory in biology. Discussions are sometimes rather lively, and the group is known for its low noise level and interesting conversation. I post the FAQ (Frequently Asked Questions) information every month.

**AIDS research**

0.5% (1 day) In collaboration with George Pavlakis and Ralf Schneider, I have been analyzing mutant HIV sequences to predict the effects on splicing.

**Publications:**

Herman ND, Schneider TD. High information conservation implies that at least three proteins bind independently to F plasmid incD repeats. *J Bact* 1992;174:3558-3560.

Rudd KE, Schneider TD. Compilation of *E. coli* ribosome binding sites. In: Miller J, ed. *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press 1992;17.19-17.45.

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Schneider TD. Use of Information Theory in Molecular Biology In: Matzke D, ed. *Workshop on Physics and Computation PhysComp '92, Proceedings of the Workshop on Physics and computation*. Los Alamitos, CA: IEEE Computer Society Press 1993:102-110.

Shaner MC, Blair IM, Schneider TD. Sequence Logos: A Powerful, Yet Simple, Tool. In: Mudge TN, Milutinovic V, Hunter L, eds. *Proceedings of the Twenty-Sixth Annual Hawaii International Conference on System Sciences, Volume 1: Architecture and Biotechnology Computing*. Los Alamitos, CA: IEEE Computer Society Press 1993:813-821.

Papp PP, Chatteraj DK, Schneider TD. Information Analysis of Sequences that Bind the Replication Initiator RepA. *J Mol Biol*, in press.

Rogan PK, Salvo JJ, Stephens RM, Schneider TD. Visual display of sequence conservation as an aid to taxonomic classification using PCR amplification. In: Pickover C, ed. *Speculations in Science and Technology and The Visual Display of Biological Information, 1993*. Teaneck, New Jersey:World Scientific 1993, in press.

Schneider TD. Genetic patterns as shown by sequence logos. In: Pickover C, ed. *Pattern Book*. Teaneck, New Jersey:World Scientific, 1993, in press.

Schneider TD. Protein patterns as shown by sequence logos. In: Keller PR, ed. *Visual Cues - Practical Data Visualization*. Piscataway, New Jersey:IEEE Press 1993, in press.

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

PROJECT NUMBER  
 Z01 CB 08397-02 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Conformational and Protein Binding Analyses of Oligosaccharides

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Pradman K. Qasba, Ph.D. Research Chemist LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Petety Balaji, Ph.D. Visiting Fellow LMMB, NCI  
 Vallurupalli S. R. Rao, Ph.D. Visiting Scientist LMMB, 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 STAFF 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.)

Diverse oligosaccharide structures that are generated with the help of specific glycosyltransferases from a relatively small number of saccharide units, have an enormous potential for encoding information. This information content for a given oligosaccharide is increased furthermore by the existence of several conformers which exist in equilibrium in solution that have been implicated in several biological functions. Most of the experimental studies in solution give a time averaged conformation, closely resembling the global energy minimum conformer, that however, need not be the bioactive conformer. To have a precise idea about the bioactive conformer, it is essential to have the information about all the conformers which are accessible by this particular oligosaccharide. We have applied molecular dynamics technique to study all the possible conformations of some bi- and triantennary oligosaccharides that are the ligands of glycosyltransferases and of asialoglycoprotein cell surface receptor (ASGP-R). Most of the earlier studies on the conformational behavior of oligosaccharides were limited to the study of individual di- and trisaccharide fragments which constitute the actual oligosaccharides present on glycoproteins. Molecular dynamics simulation of the molecules for one nano second by considering all the monosaccharides simultaneously has provided a wealth of information about the conformational preferences of these molecules. The results show that contrary to earlier beliefs oligosaccharides show considerable amount of flexibility. The relative distance and orientation of the galactose residues on the 1,2- and 1,4-branches of the 1,3-arm of triantennary oligosaccharide are invariant during most of the simulation period. Contrary to the 1,3 arm, the 1,6 arm is close to the GlcNAc residues of the core oligosaccharide structure. Binding of the 1,3-arm may bring about a conformational change in the 1,6-arm and lead to its interaction with a lectin, e.g ASGP-R, providing additional binding energy.

## PROJECT DESCRIPTION

Objective:

1. Conformational analysis of oligosaccharide structures, which are ligands for glycosyltransferases, lectins and cell surface receptors, by molecular dynamics simulation
2. To identify by molecular modeling methods the residues and any alterations in the  $\alpha$ -C atom backbone of  $\alpha$ -lactalbumin which blocks the binding of sugar moiety into the site equivalent to that of sugar binding site of structurally homologous protein lysozyme

In solution several conformers of an oligosaccharide exist in equilibrium. Most of the experimental studies in solution give a timed average conformation, that closely resembles the global energy minimum conformer, which need not be the bioactive conformer. To have a precise idea about the bioactive conformer, it is essential to have information about all the conformers which are accessible by this particular oligosaccharide. Molecular dynamics (MD) simulations of oligosaccharides are being used to resolve many questions of direct biological relevance. In this study, molecular dynamics technique has been applied to study all the possible conformations of some of the ligands of glycosyltransferases and of important cell surface receptors.

Major Findings:**Flexibility of the oligosaccharide**

Classically oligosaccharides have been thought to be relatively "rigid" molecules meaning they show little variability in their conformations. In bi- and triantennary oligosaccharides whatever little flexibility exists, it was associated with the 1,6 linkage since there are three bonds around which rotations can be made in a 1,6 linkage. But results from the present study show that oligosaccharides are "flexible" molecules showing significant conformational variability. This is clearly reflected in the plots of inter-galactose distances versus time. From nmr studies on di- and trisaccharides, it has been hypothesized that the hydrogen bond between Man4:HO3 and GlcNAc7:O5 introduces a high degree of segmental constraint and hence decreases the flexibility of N-acetyllactosamine type complex oligosaccharides. Molecular simulation studies from triantennary oligosaccharide show that the variation in distance with time is more for the Gal residues on the 1,3-arm compared to the Gal residue on the 1,6-arm indicating that the 1,3-arm is much more flexible than the 1,6-arm. This flexibility for the 1,3-arm Gal residues mainly arises from the  $\alpha$ 1,3-linkage between Man3 and Man4 and the  $\beta$ 1,2-linkage between Man4 and GlcNAc6.

### Importance of $180^\circ, 0^\circ$ conformation for $\beta 1,4$ and $\beta 1,2$ linkages

Although the torsion angles  $\phi, \psi$  between GlcNAc- $\beta 1,2$ -Man fluctuate around some of the minimum energy conformations most of the time,  $\phi$  shows transitions to  $180^\circ$  also. This conformation also corresponds to a local minimum from force field calculations on disaccharide fragments. The present dynamics study shows that the energy barrier between these conformations can be easily crossed. This conformational transition to  $\phi = 180^\circ$  leads to 'flipping' of the N-acetyllactosamine group and this may be important for certain oligosaccharides for recognition by the respective receptors. Such a conformation has indeed been observed for the  $\beta 1,2$  linkage in the crystal structure of the human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus*. This type of conformational change has been seen for the  $\beta 1,4$  linkage also. But such a possibility was not considered while proposing models for complex oligosaccharides earlier. The terminal Gal residues which are recognized by the asialoglycoprotein receptor (ASGP-R) are connected to GlcNAc by a  $\beta 1,4$  linkage. Hence it is possible that galactose may 'flip' when the triantennary oligosaccharide binds to the receptor.

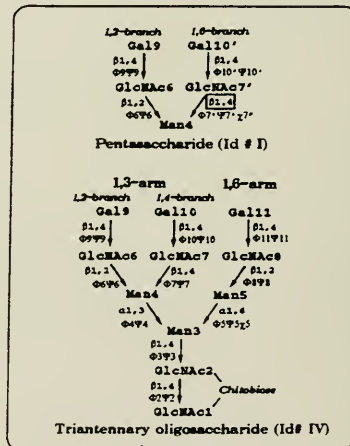
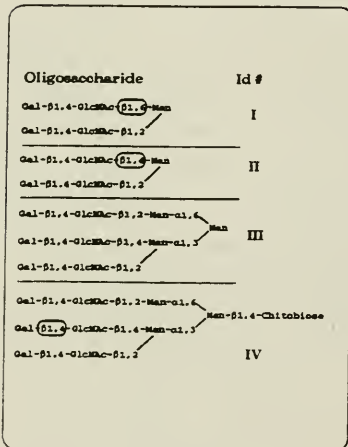
### Effect of the chitobiose core on the conformation of the triantennary oligosaccharide

Oligosaccharide IV differs from III in having an chitobiose core attached to Man3 in a  $\beta 1,4$ -linkage. GlcNAc1, GlcNAc2, Man3, Man4 and Man5 constitute the classic core seen in all the N-linked glycoproteins. Molecular dynamics simulation of both the oligosaccharides were done with nearly the same starting conformations and with all the three staggered conformations for  $\chi 4$ . Behavior of all the torsion angles in the 1,3-arm was found to be very similar in the two molecules implying that the presence or absence of the chitobiose does not drastically affect the conformational preferences of the 1,3-arm. However, conformation of the 1,6-arm showed a remarkable difference in the two triantennary oligosaccharides. In the absence of the chitobiose core, 1,6-arm was found to be closer to the 1,2- and 1,4-branches of the 1,3-arm due to preference of  $60^\circ$  conformation for  $\chi 4$ . However, this is not the case when the chitobiose core is present on Man3. Generally, in oligosaccharide IV, the 1,6-arm tends to come closer to the chitobiose core.

### Binding affinities of pentasaccharides I and II

Pentasaccharide I differs from II in having a  $\beta 1,6$  linkage instead of  $\beta 1,4$  linkage between Man4 and GlcNAc7. However, pentasaccharide II shows nearly 15-fold higher affinity to ASGP-R than pentasaccharide I. According to the 'golden' triangle hypothesis, the affinity of ligands to ASGP-R depends on the number of Gal residues present in the ligand and the distance separating them. Both pentasaccharides I and II have two terminal Gal residues. Molecular dynamics simulations of these oligosaccharides have shown that the average distance between the terminal Gal residues in both I and II is about the same. It thus seems that the factors considered in the 'golden' triangle hypothesis,

i.e., the number of terminal Gal residues and the distance between them alone is not sufficient to explain the experimental data. The common Man4-GlcNAC6-Gal9 trisaccharide fragment of pentasaccharide I superposed over that of II shows that although Gal9 is in the same orientation in both the oligosaccharides, Gal10 and Gal10' are placed differently in the two pentasaccharides. Hence while binding to the two Gal binding sites of the ASGP-R, if Gal9 occupies one of the binding site, then Gal10' in pentasaccharide I can not occupy the second binding site which is accessible to Gal10. Hence ASGP-R can bind to only Gal9 or Gal10' of pentasaccharide I and not to both of them simultaneously leading to weak binding compared to II. Thus the present molecular dynamics studies suggest that the proper distance between the Gal residues is a necessary but not sufficient condition. Recognition of the oligosaccharide by the receptor requires, in addition to the proper separation, correct orientation of the Gal residues with respect to one another.



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

PROJECT NUMBER

Z01 CB 08399-01 LMMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 CHARACTERS OR LESS. TITLE MUST FIT ON ONE LINE BETWEEN THE BORDERS.)

Functional Analysis of the Catalytic Domain of  $\beta$ 1-4galactosyltransferase

PRINCIPAL INVESTIGATOR (LIST OTHER PROFESSIONAL PERSONNEL BELOW THE PRINCIPAL INVESTIGATOR.) (NAME, TITLE, LABORATORY, AND INSTITUTE AFFILIATION)

Pradman K. Qasba, Ph.D.                      Research Chemist                      LMMB, NCI

OTHER PROFESSIONAL PERSONNEL:

Elizabeth Boeggeman, Ph.D.                      Senior Staff Fellow                      LMMB, NCI  
 Petety Balaji, Ph.D.                      Visiting Fellow                      LMMB, NCI  
 Arni Masibay, Ph.D.                      Staff Fellow                      LMMB, 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 STAFF YEARS:

1.75

PROFESSIONAL:

1.75

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.)

Bovine  $\beta$ 1,4galactosyltransferase ( $\beta$ 1,4GT) is a 402 residue long protein, which has a general topology similar to other glycosyltransferases that consists of a short amino-terminal cytoplasmic tail, a membrane signal anchor domain, a stem region and a carboxyl-terminal catalytic domain. The enzyme transfers galactose from UDP-galactose to N-acetylglucosamine (NAGlc), either free or bound to an oligosaccharide, to produce N-acetylglucosamine with a  $\beta$ 1-4 linkage. To analyze the catalytic domain we prepared cDNA constructs of the amino-terminal deleted forms of the protein in pGEX-2T vector and expressed them in *E. coli* as glutathione-S-transferase (GST) fusion proteins. Recombinant proteins were localized in inclusion bodies which were solubilized in 5 M guanidine-HCl. Renaturation and regeneration of the enzyme activity, from the solubilized protein was strictly dependent on the presence of an "oxido-shuffling" reagent, a mixture of 8:1 mM reduced:oxidized glutathione. Renatured/reoxidized fusion proteins, purified on glutathione-affinity columns, were active in  $\beta$ 1,4GT and lactose synthetase (LS) assays. After thrombin cleavage of the fusion protein and subsequent removal of GST domain, the recombinant  $\beta$ 1,4GT has 50-85% of the specific activity of bovine milk GT. The apparent  $K_m$ 's for NAGlc and UDP-galactose were similar to that of bovine milk  $\beta$ 1,4GT. Deletion analysis show that both  $\beta$ 1,4GT and LS activities remain intact even in the absence of the first 129 residues, but the activities are lost when deletions extend up to residue 142. Site directed mutagenesis of Cys 134 to either Ala or Ser resulted in the loss of both  $\beta$ 1,4GT and LS enzyme activities. In contrast to the previously reported result (Aoki D, Appert HE, Johnson D, Wong SS, Fukuda MN (1990) EMBO J. 9, 3171-3178), our results show that  $\beta$ 1,4GT produced in *E. coli* is present in inclusion bodies as an inactive protein. "Oxido-shuffling" reagents are required for the formation of a disulfide bond involving Cys 134, which is crucial for proper folding of the protein and for the regeneration of the enzyme activity.

## PROJECT DESCRIPTION

Objective:

To determine: a) the maximum length of the "stem" region of  $\beta$ 1,4galactosyltransferase protein that can be dispensed without effecting the catalytic function of the enzyme; and b) to produce the protein in large quantities for the 3-D structure determination

Analyses of the protein sequences of glycosyltransferases, derived from their cDNA sequences, have suggested that the catalytic domain of these enzymes lie in the carboxyl-terminal portion of the protein. The objective of the present studies is to determine by the recombinant DNA methodology and by expressing the protein in *E. coli*; a) the length of the stem region that can be deleted and still retain the enzymatic function of the protein, b) identify the residues that are essential for the catalytic function of the protein, and c) to produce the protein in large quantities for 3-D structure determination.

Major Findings:Construction and expression of 5'-end deleted  $\beta$ 1,4GT cDNAs

To construct the pGT-d75 vector that would code for the soluble form of  $\beta$ 1,4GT found in milk, a 1.59 Kb Sst I-EcoR I fragment of pLbGT-1 (Narimatsu et al., 1986) was subcloned into the polylinker site of pSPT18. A clone, SPT18-S/E-GT, was identified and characterized which contains sequence coding for amino acids 76-402 of  $\beta$ 1,4GT and a portion of the 3'-non-coding sequence. A 1.6 Kb  $\beta$ 1,4GT fragment of pSPT18-S/E-GT that had a BamH I and EcoR I site at the 5'- and 3'-ends, respectively, was subcloned into the BamH I/EcoR I site of pGEX-2T generating the construct pGT-d75. The vector pGEX-2T carries sequences that encode the 26 kDa glutathione binding domain of GST. It also codes for thrombin recognition sequence at the carboxyl-terminus of GST and has BamH I/EcoR I restriction sites for subcloning of DNA fragments. The other amino-terminal deletion mutants of  $\beta$ 1,4GT were constructed using PCR technique to amplify DNA sequences contained in pLbGT-1. The bacterial cultures harboring the deleted and mutated construct were grown, induced with IPTG, harvested and lysed. The supernatant (soluble) and the pellet (insoluble protein) fractions were separated by centrifugation and analyzed on SDS gels. The fusion proteins were mainly present in the insoluble pellet fraction.

## Requirements for renaturation of recombinant protein from inclusion bodies

The inclusion bodies were solubilized in 5 M guanidine-HCl. Several conditions for the renaturation of recombinant proteins were tested. An aliquot of each sample was diluted ten fold in a buffer that contained various agents. Renaturation and regeneration of  $\beta$ 1,4GT activity was absolutely dependent on a mixture of reduced and oxidized glutathione. The maximal activity was obtained



when the renaturation buffer contained at least 1% Triton X-100, reduced and oxidized glutathione in a ratio of 8:1 mM, and the pH of the solution was 8.0. Glycerol concentration above 5% was found to interfere with the refolding process. Neither MnCl<sub>2</sub> nor UDP-galactose are required during the renaturation process to regenerate the enzyme activity. Treatment of the solubilized inclusion bodies with DTT, prior to renaturation, did not increase  $\beta$ 1,4GT activity. The renatured samples showed a two fold increase in enzyme activity after dialysis. SDS-gel analysis of the dialyzed samples show that the major protein band corresponds to the fusion protein. At this stage of purification the recombinant proteins could be cleaved with thrombin releasing the GST domain of the fusion protein.

### Glutathione-affinity purification of $\beta$ 1,4gt-fusion proteins

The renatured fusion proteins with a deletions up to first 129 residues bind to glutathione-sepharose and can be purified on these columns. However, the renatured fusion protein that lacks residues 1-142 of  $\beta$ 1,4GT, shows a lower binding affinity to the glutathione sepharose column. The same was true for the mutant fusion protein Cys134Ser. The GST domain can be directly removed from the  $\beta$ 1,4GT fusion protein bound to glutathione sepharose, by treating the matrix with thrombin and then eluting the recombinant protein from the column. With the exception of GT-d142 and GT-d129C134S, the final yield of the fusion protein from deletion constructs was approximately 2 mg/l of bacterial culture. Both uncleaved and thrombin cleaved fusion proteins were further analyzed for  $\beta$ 1,4GT and LS activities.

### Enzymatic activity of deletion constructs and cys134 mutants

The fusion proteins derived from pGT-d75 and pGT-d129 were active in  $\beta$ 1,4GT and  $\alpha$ -lactalbumin dependent lactose synthetase (LS) activities. The fusion protein from GT-d142, which lacks residues 1-142 that includes Cys134, and the mutant protein Cys134Ser, show neither  $\beta$ 1,4GT nor LS enzyme activities even after removal of the GST domain from the fusion proteins. The mutants that were active prior to cleavage had a 2-3 fold increase in  $\beta$ 1,4GT activity after thrombin treatment. The GST domain at the amino-terminal end of the deletion construct partially inhibits  $\beta$ 1,4GT activity but not LS activity.

The specific activity of the recombinant protein varied from preparation to preparation and ranged between 50 to 85% of milk bovine GT. Only 50% of the recombinant protein with a specific activity of 30-40% to that of bovine milk GT bound to  $\alpha$ -lactalbumin affinity column. The eluted protein from this column showed a two fold increase in its enzymatic activity. The kinetic constants for N-acetylglucosamine and UDP-galactose for GT-d75 and GT-d129 were comparable to the bovine milk  $\beta$ 1,4GT. These results show that  $\beta$ 1,4GT deletion constructs code for proteins which have similar affinities for both donor and acceptor substrates as the native bovine milk protein. Furthermore, in the presence of Mn<sup>++</sup> both GT-d75 and GT-d129 proteins bind to UDP-Hexanolamine and GlcNAc- agarose columns and can be eluted with 5 mM GlcNAc. Residues 1-129 are

not involved in the binding of either UDP-galactose or N-acetylglucosamine but disruption of the disulphide bond involving C134 affects the binding of the protein to its substrates.

**Publications:**

Boeggeman EE, Balaji PV, Sethi N, Masibay AS, Qasba P K. Expression of deletion constructs of bovine  $\beta$ 1-4galactosyl-transferase in *E.coli*: Importance of Cys 134 for its activity. Protein Engineering, in press.

SUMMARY REPORT  
LABORATORY OF PATHOLOGY  
DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS  
NATIONAL CANCER INSTITUTE  
1993

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 11 sections:

Surgical Pathology Section (Dr. Maria J. Merino, Chief)  
Pulmonary Pathology Section (Dr. William D. Travis, Chief)  
Postmortem Pathology Section (Dr. Gitie S. Jaffe, 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)  
Molecular Pathology Section (Dr. Mark E. Sobel, 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

The Surgical Pathology Section provides expertise and diagnostic services in the field of Anatomic Pathology for the Institutes and Clinical Center patients, and collaborates with the research staff in those investigations which involve the use and study of human pathological material. Approximately 6,000 surgical specimens and biopsies (more than 60,000 slides which include routine and a variety of special stains) were accessioned last year. These include more than 2,000 fresh human tissues. A tissue procurement nurse works in close collaboration with the surgical pathology staff and helps in the distribution of tissues to scientists throughout the NIH.

The members of the section also participate in a variety of teaching and interdepartmental conferences (Medicine Branch, Surgery Branch, etc.) in which patient diagnosis and modalities of therapy are discussed, assisting in this way, to provide better patient care. Other objectives of the Surgical Pathology Section include carrying independent research by the members of the section and providing a residency program in anatomic pathology.

The section also provides consultant services to the community as well as to pathologists throughout the country.

Dr. Merino, in collaboration with other members of the Surgical Pathology staff, is investigating the role of different tumor markers as prognostic tools in the diagnosis of breast, ovarian and thyroid cancer, as well as soft tissue sarcomas. Dr. Merino is currently evaluating a number of antibodies used as prognostic indicators of breast cancer (p53, cerb-2, EGFR), antibodies against enzymes known to be important in progression to tumor invasion and metastases (collagenases and cathepsins), and antibodies that facilitate the recognition of breast cancer in distant sites (GCDFP-15). Her goal is to find specific markers that can predict aggressive behavior, early recurrences, and response to therapy. The section is also investigating the use of antibodies against P-glycoprotein, which has been associated with a multidrug resistant phenotype; its presence is being evaluated in breast, ovarian and endometrial cancers as well as normal, endometrial tissues.

Dr. Merino is currently doing a study on breast cancer in young premenopausal women. The study will include a comprehensive investigation of identified histologic precursors as well as an immunohistochemical evaluation of prognostic markers such as p53, cerb-2 and nm23.

#### Pulmonary Pathology Section

Dr. William 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. The molecular biology of lung cancer is being studied based on a group of prospectively collected cases correlating results with clinical, epidemiologic and prognostic features. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings. A new fellowship has been established for training in pulmonary pathology.

#### Postmortem Pathology Section

Dr. Gitie Jaffe, in collaboration with members of the surgery branch, is studying cytokine secretion by genetically modified nonimmunogenic fibrosarcomas. In addition, Dr. Gitie Jaffe is reviewing the renal pathology of a familial form of renal cell carcinoma associated with Von Hippel-Lindau disease.

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, immunohistochemical, molecular and flow cytometric techniques are being applied to the study of these diseases.

Cytopathology Section

The Laboratory of Pathology provides complete services in anatomic pathology for the Clinical Center, a 550 bed research hospital. The Cytopathology Section provides diagnostic service on exfoliative cytology, fine needle aspiration cytology, and immunocytochemistry. The section accessions approximately 3,500 specimens per year. The relatively high rate of pathologic findings combined with the diversity of types of exfoliative and FNA specimens, provide a broad experience in diagnostic cytopathology for residency training.

Cytopathology research efforts most recently have been directed towards the application of immunocytochemistry in diagnostic cytopathology. Lymphoid markers have been utilized to differentiate reactive processes from lymphoma, as well as to subtype lymphomas when possible. Several monoclonal antibodies have been evaluated for specificity and sensitivity for carcinoma cells versus reactive mesothelial cells in the diagnosis of metastatic carcinoma in cavity fluids. Currently under investigation is the use of *in situ* hybridization as an ancillary diagnostic technique for cytology.

Through a national consensus conference, this section has established the "Bethesda" system for standardizing the classification and reporting of PAP smears.

Ultrastructural Pathology Section

A. Diagnostic Services

1. Diagnostic electron microscopic services are provided to a diverse group of Clinical Center physicians at the NIH. Approximately 200 cases are accessioned every year; over 150 cases are completed and supplemental EM reports are issued.

2. Specialized diagnostic services on solid pediatric tumors are provided to the Pediatric Oncology Branch (POB) at the NCI. The section participates in a weekly Tumor Board for discussion of pediatric cases.

B. Research Interests

The section, under Dr. Maria Tsokos, studies the biology of solid pediatric tumors in general, and especially those that are grouped under the term "small round cell tumors of childhood". Current research projects in the section include:

1. Identification of p53 gene mutations in rhabdomyosarcoma, Ewing's sarcoma and primitive neuroectodermal tumors (PNET) on PCR-amplified DNA from paraffin blocks using mutation detection enhancement (MDE) gels. The presence of mutations is verified by direct DNA-sequencing. Presence or absence of 53 mutations will be correlated with clinical outcome.

2. Detection of specific fusion transcripts in tumors with 11;22 translocation by reverse transcriptase-PCR and evaluation of the method as a diagnostic tool in the every day practice.

3. Role of growth factors and their receptors in the development and differentiation of pediatric tumors. The investigated factors are Transforming Growth Factor (TGF)- $\alpha$  and  $\beta$  and Epidermal Growth Factor (EGF). Those studies consist of: (a) immunohistochemical staining of paraffin sections with appropriate antibodies and (b) in vitro evaluation of cell growth and differentiation after addition of the growth factor, growth factor levels of expression before and after differentiation (Northern analysis), possible DNA amplification (Southern analysis), and affinity of the growth factor for its own receptors (binding assays). TGF protein levels are also measured in tissue culture media with an enzyme-linked immunosorbent assay and CCL-64 mink lung cell growth inhibitory assay. The ultimate goal of these studies is to create a model for investigating pathogenetic mechanisms and identifying factors that may lead to potential therapeutic trials.

4. Immunohistochemical studies with the 12E7 antibody against antigenic surface determinants of Ewing's and PNET cells in comparison with antibodies to the muscle determination gene MyoD<sub>1</sub> to evaluate specificity and sensitivity of these markers in the differential diagnosis of very primitive rhabdomyosarcoma from Ewing's sarcoma and PNET.

5. Immunohistochemical studies with antibodies against proliferating cell nuclear antigens (PCNA) and comparison of levels of expression with the S-phase of the cell cycle, mitotic index and clinical outcome. The S-phase will be calculated by flow cytometric analysis with a FACSCAN flow cytometer equipped with a 15- mW 488- nm Argon laser.

#### Biochemical Pathology Section

The Biochemical Pathology Section, under Dr. David Roberts, is conducting research on the role of the adhesive glycoprotein thrombospondin in tumor growth and metastasis and the host receptors mediating adhesion of pathogenic microorganisms. Cell-cell and cell-matrix interactions are important regulators of normal cell growth and differentiation and play essential roles in pathological conditions including tumor metastasis and initiation of infection by many pathogens. Defining the molecules mediating adhesion and their cell surface and matrix receptors is a prerequisite for designing pharmacological agents to inhibit these processes. Current research projects in the section include: 1) identification of tumor cell receptors for thrombospondin and characterization of the signal transduction pathways

mediating responses to thrombospondin binding to these receptors; 2) identification of peptide sequences in thrombospondin mediating tumor cell adhesion and migration; 3) characterization of the interactions of thrombospondin and laminin with sulfated glycolipids and proteoglycans and their role in regulation of angiogenesis, tumor growth, and metastasis; and 4) determination of the molecular mechanism for interaction of pathogens with host epithelial cells and extracellular matrix components.

Two regions of the thrombospondin molecule have been identified that mediate adhesive and migratory responses of cultured human melanoma cells to thrombospondin. The carboxyl-terminal domain mediates attachment and haptotaxis, and the amino-terminal domain mediates cell spreading and chemotaxis. The cell receptors recognizing these two regions of thrombospondin are under investigation. Sulfated glycoconjugates, including heparan sulfate proteoglycans and sulfated glycolipids, interact with the amino-terminal domain of thrombospondin. An unusual sulfated glycolipid, present only in melanoma cell lines that spread on thrombospondin, binds to thrombospondin and participates in melanoma cell spreading on thrombospondin but not on fibronectin. Integrin and non-integrin receptors for the carboxyl-terminus of thrombospondin are being characterized in several types of tumor and normal cells. Peptides from the type I repeats of thrombospondin were identified that mimic the activities of the whole molecule for regulating cell adhesion, migration, and proliferation. Two active sequences were identified in this region. The consensus sequence Trp-Ser-Xaa-Trp binds to heparin, promotes cell adhesion and motility, and inhibits proliferation. A peptide from the second type I repeat binds specifically to fibronectin and inhibits fibronectin-mediated interactions of cells with type I collagen.

The intracellular responses of cells binding to each active domain or sequence of thrombospondin are being investigated. Exposure of melanoma cells to thrombospondin induces transient changes in cyclic nucleotide and inositol phosphate levels. Based on sensitivity to agents that modify cyclic AMP levels, chemotaxis of melanoma cells in a thrombospondin gradient is mediated by changes in cAMP levels and a pertussis toxin-sensitive G protein. Based on its effects on tumor cell adhesion, growth, and motility, expression of thrombospondin by tumor cells may regulate their metastatic phenotype. Thrombospondin mRNA and protein expression were decreased in subclones of K1735 melanoma cells selected for high metastatic potential in mice and in human lung epithelial cell lines transfected with ras and selected for tumor formation by growth in nude mice.

Several approaches are being used to characterize the interactions of sulfated glycoconjugates with the adhesive proteins thrombospondin and laminin. Fragments or peptides from both proteins were identified that bind specifically to heparin or sulfatide. A proteolytic fragment containing 394 amino acids from the carboxyl-terminus of the A chain of laminin bound specifically to sulfatide. Binding of this fragment is probably mediated by clusters of basic amino acid residues. Similar basic consensus sequences occur in the amino terminal domain of thrombospondin. In contrast, binding of the type I repeats of thrombospondin to heparin does not require basic amino

acids and is mediated by a novel heparin binding sequence containing two tryptophan residues. Using defined oligosaccharides from heparin, the two heparin binding sequences from thrombospondin were shown to have different binding specificities. A similar tryptophan-containing peptide from a 33 kDa protein related to the 67 kDa laminin receptor also bound to heparin and inhibited heparin-dependent interactions of laminin with cells. Adhesion of endothelial cells but not melanoma cells on a laminin substrate is dependent on proteoglycans on the cell surface, based on inhibition of adhesion by the sulfation inhibitor chlorate.

Recognition of host cell surface glycoconjugates or extracellular matrix proteins is a critical early step in initiation of infection by pathogenic microorganisms. Adhesive specificities of some pathogenic bacteria and the yeast *Candida albicans* are being examined. Binding of *C. albicans* to fibronectin is mediated by several domains of the protein. The gelatin-binding domain of fibronectin contains the highest affinity binding site for *C. albicans*. Inhibitors of each binding specificity will be identified using solid phase assays and then tested using *in vitro* cytoadherence assays and *in vivo* infection assays to determine the role of each in cytoadherence and initiation of infection.

#### Tumor Invasion and Metastases Section

Invasion and metastasis, the most life-threatening aspect of cancer is the culmination of a series of progression steps resulting in genetic changes over and above those required for uncontrolled proliferation. Expression of the metastatic phenotype depends on a balance between positive and negative regulatory gene products. Understanding the action of these gene products has led to new strategies for prognosis and therapy.

Dr. William Stetler-Stevenson is studying type IV collagenase, a metalloproteinase first identified by this section, which cleaves basement membrane type IV collagen at a specific locus, and is augmented in metastatic tumors. Negative regulation of type IV collagenase may be mediated through TIMP-2, a novel human metalloproteinase inhibitor identified and cloned by Dr. Stetler-Stevenson. The complete domain structure of TIMP-2 has been determined, and the chromosomal location of TIMP-2 on 17q has been determined and confirmed. TIMP-2 binds to the latent form of type IV collagenase with a one-to-one molar stoichiometry and abolishes the catalytic activity of the activated enzyme. TIMP-2 may function as a tumor suppressor protein by inhibiting metalloproteinase activity required for invasion. TIMP-2 totally blocks the invasion of cancer cells through reconstituted basement membranes *in vitro*. *In vivo* TIMP-2 arrests metastasis through inhibition of angiogenesis. Specific clinical applications of TIMP-2 could include the treatment of bone metastasis, because bone destruction is mediated by collagenases.



Progression to the metastatic phenotype may involve the loss of genes normally involved in development, morphogenesis or differentiation. Dr. Patricia Steeg, another investigator in the section, identified the nm23 gene family. Expression of nm23 is reduced in highly metastatic human breast, hepatocellular and melanoma tumors; in metastatic colorectal and neuroblastoma tumors nm23 mutations have been identified. The 17 kDa protein product of nm23 is virtually identical to the awd protein involved in *Drosophila* development and morphogenesis. Expression of nm23 was associated with the functional differentiation of multiple epithelial tissues in mouse embryogenesis.

Transfection of murine nm23-1 into murine K-1735 TK melanoma cells and human nm23-H1 into human MDA-MB-435 breast carcinoma cells has been reported. In each case, expression of nm23 resulted in a significant decrease in metastatic potential *in vivo*. Recent studies indicate that nm23 protein has multiple biochemical activities, including a nucleoside diphosphate kinase activity and a cAMP regulated ATPase activity. The role of each biochemical activity in nm23 function is under investigation. As a cancer marker, nm23 may provide a new approach to predicting the metastatic aggressiveness of an individual patient's tumor. Agents which modulate nm23 expression or function, or mimic its action, may have therapeutic potential.

Locomotion is a necessary component for tumor cell invasion. Members of the section have also been studying the transducer systems involved in the stimulated motility of invasive cancer cells. Dr. Mary Stracke is cloning the gene for a potent new motility stimulating cytokine, autotaxin. Autotaxin is a 120,000 dalton glycoprotein that has recently been purified and partially sequenced from the human melanoma cell line, A2058. This cytokine stimulates a pertussis toxin-sensitive motility response in these same cells at concentrations from 100 pM to 20 nM. Anti-peptide antibodies have been produced in rabbits against selected autotaxin peptides that recognize the protein on immunoblots. These affinity-purified antibodies are being utilized for biochemical and histochemical studies of autotaxin. Dr. Beckner has cloned the gene for a new transmembrane protein which regulates tumor cell locomotion. Cytokine mediated stimulation of human melanoma cell motility was found by Dr. Aznavoorian and Dr. Savarese to operate through a pertussis toxin sensitive G protein pathway which regulates arachadonic acid and calcium fluxes. Dr. Aznavoorian has developed a new system to measure and isolate individual pseudopodia. This has led to new insights into the mechanism of pseudopodial protrusion and the role of G proteins, cytoskeleton and receptors in this process.

Screening compound which inhibits this specific pathway has led Dr. Kohn to identify a new signal transduction inhibitor which blocks tumor cell cytokine stimulated growth and motility. The inhibitor, termed CAI, is a substituted triazole which constitutes a new approach to cancer therapy. In animal models using a variety of human tumors, including melanoma, CAI has produced primary tumor and metastasis regression following oral administration. CAI has low toxicity, in studies to date, and is being considered as a potential chemopreventive agent. Clinical phase I trials for

treatment of refractory cancers began in March, 1992. Low toxicity and promising tumor responses have been seen in the first 14 patients.

### Molecular Pathology Section

The Molecular Pathology Section develops and coordinates the training of residents, intramural staff, and extramural scientists on the application of molecular biology techniques to the study of cancer biology, providing a resource center for the analysis of gene expression in neoplasia. A variety of techniques are utilized, including expression cDNA cloning, DNA sequencing, genomic DNA isolation and analysis, polymerase chain reaction, ligase chain reaction, restriction endonuclease digestion of DNA, RNA extraction from tissues and *in vitro* cell lines, tissue and cellular protein extractions, RNA blot hybridization, *in situ* hybridization, development of anti-synthetic peptide antibodies for immunoblot and immunohistochemical analysis, DNA transfections, and development and labeling of hybridization probes including DNA, oligonucleotide, antisense RNA, and sense RNA. Training is provided at the laboratory bench as well as through weekly molecular biology journal clubs and data sessions.

Research interests of the section include the expression and characterization of extracellular matrix receptors on cancer cells and the expression of homeobox genes in human cancers. The section has cloned three non-integrin laminin binding proteins that are expressed on the surface of normal and cancer cells. Immunohistochemical, immunoblot, RNA blot, and RNA *in situ* hybridization studies have demonstrated that expression of a 67 kilodalton laminin receptor is increased in a variety of human cancers, especially breast, colorectal, gastric, and ovarian. In contrast, expression of a 31 kilodalton laminin binding protein with lectin binding properties is decreased at both the protein and RNA levels in colorectal adenocarcinomas. The physiologic significance of this inverse modulation of expression of two non-integrin laminin binding proteins is being studied by sense and antisense transfection studies. In addition, a 14 kilodalton laminin binding protein has been cloned in the section and its expression is currently under investigation. Using cDNA-derived synthetic peptides and anti-synthetic peptide antibodies, domains of the laminin binding proteins have been defined. The 67 kilodalton laminin receptor is part of a multigene family containing many pseudogenes. In the course of searching for the regulatory regions responsible for active transcription of the gene, several pseudogenes have been identified.

Homeobox genes, encoding transcriptional regulators, act in complex regulatory cascades to control the coordinated expression of genes involved in specific developmental processes. Originally identified and studied in *Drosophila*, homeobox genes have now been isolated from a variety of vertebrate species, including human. The Molecular Pathology Section is determining whether specific homeobox genes may control the coordinated expression of genes involved in human cellular transformation and in tumor invasion and metastasis. Human breast cancer has been targeted as an initial model system.

The approach takes advantage of the fact that all known homeobox genes in all species share a common 183 bp segment of DNA that encodes a highly conserved 61 amino acid domain responsible for binding to DNA. Using degenerate primers within this common DNA segment and polymerase chain reaction technology, homeobox gene segments were amplified from RNA that had been extracted from a variety of human breast cancer cell lines. Subsequently, these sequences were used to probe a cDNA library derived from the metastatic human breast cancer cell line MCF7. Six homeobox cDNA clones were isolated and characterized. Several contain base mutations, and one clone contains an insertion. The physiologic significance of these mutations will be assessed by DNA transfection studies.

### Hematopathology Section

The Hematopathology Section conducts a major program in diagnostic and experimental hematopathology. The section offers expertise in the diagnosis of hematopoietic disorders for patients admitted to the National Institutes of Health. The staff collaborate closely with physicians treating patients with neoplastic and reactive hematologic and lymphoproliferative disorders. While the emphasis is on clinical protocols based in the NCI, collaborations exist with physicians in NIAID, NHLBI, NEI, and NIAMS. Dr. Jaffe supervises an internationally recognized consultation service which receives over 1200 cases per year in consultation from the medical community.

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, the Biological Response Modifier Program in Frederick, Maryland, NCI, and the Metabolism Branch, NCI.

Immunophenotypic analyses are performed using frozen and paraffin section immunohistochemistry and flow cytometry. The flow cytometry laboratory utilizes a FACS scan and a FACS star, and is supervised by Dr. Maryalice Stetler-Stevenson. The section also offers studies in applied molecular diagnosis, using DNA and RNA probes. The diagnostic molecular biology laboratory is supervised by Dr. Mark Raffeld. These facilities are all integrated in the fellowship program in hematopathology.

The Hematopathology Section has published a number of important studies on the clinicopathologic and immunophenotypic aspects of malignant lymphoma. Dr. Jaffe described a unique association of nodular lymphocyte predominant Hodgkin's disease (NLPHD) and co-existent large cell lymphoma. In contrast to what would be expected for large cell lymphoma, all patients had localized disease clinically and 6 of 7 achieved long-term, disease-free survival. Following this observation, a Registry was established for the compilation of

this entity and future study. At present, more than 40 cases have been submitted to the Registry. Work in progress using polymerase chain reaction (PCR)-based technology is examining the clonality of the large cell lymphoma component as well as the antecedent NLPHD.

The section has expanded its analyses of the angiocentric immunoproliferative lesions. Evidence of an association with the Epstein-Barr virus has been extended to include a cluster of cases in Peru. As described previously in Asian countries, angiocentric immunoproliferative lesions in Peru frequently present with midline destructive nasal disease. EBV was identified by *in situ* hybridization in all cases expressing a T-cell phenotype.

In a series of papers, the interrelationship between Hodgkin's disease and the non-Hodgkin's lymphomas was explored by studying cases in which both diseases are present in the same patient, either as composite lymphomas or as sequential or simultaneous Hodgkin's disease and non-Hodgkin's lymphoma. Based on these studies, it was observed that Hodgkin's disease and non-Hodgkin's lymphomas occur together with greater frequency than would be expected by chance alone. Moreover, the association favors a lymphoid origin for the malignant cell of Hodgkin's disease, and given the predominance of B-cell non-Hodgkin's lymphoma, would be consistent with a B-cell origin in the majority of cases. The studies also suggest a clonal relationship between certain forms of Hodgkin's disease and non-Hodgkin's lymphoma and suggest that usual or classical Hodgkin's disease may be an altered lymphoid malignancy, with secondary transformation by a virus such as Epstein-Barr virus and/or other candidates yet to be identified. For example, in studies of Hodgkin's disease occurring in association with chronic lymphocytic leukemia, EBV was demonstrated by *in situ* hybridization in the Reed-Sternberg cells and mononuclear variants, but not in the associated chronic lymphocytic leukemia lymphocytes.

Dr. Jaffe also completed an analysis of the lymphoid lesions seen in the common variable immunodeficiency syndrome. While the incidence of non-Hodgkin's lymphoma in CVID has been reported to range from 30-fold to 438-fold, the risk may have been overestimated in the past due to the misdiagnosis of atypical lymphoid hyperplasia as malignant lymphoma. Following immunophenotypic and molecular analyses, the majority of lymphoid lesions occurring in CVID are reactive and can be classified as atypical lymphoid hyperplasia or reactive lymphoid hyperplasia. Two patients developed immunoblastic lymphomas, one of which was demonstrated to be associated with EBV by *in situ* hybridization.

Dr. Raffeld has continued his work to define the molecular events involved in the transformation of low-grade lymphomas to more aggressive forms. In the past year he completed an analysis of the role of c-myc and found acquired structural changes in this gene in approximately 10% of progressed lymphomas. Another gene which appears to play a role in histologic progression is bcl-3 located on 17q22. Abnormalities of this locus were found in 10-15% of progressed lymphomas. In contrast to the situation for c-myc,

these changes are not temporally associated with the progression event, but may precede histologic progression and predispose to it. Finally, a study on the role of the p53 gene in progression was performed using SSCP analysis as well as immunohistochemistry. Acquired mutations of p53 could be demonstrated in approximately 1/3 of progressed follicular lymphomas, while the antecedent low-grade follicular lymphomas did not possess such mutations. It is of interest, however, that rare cells demonstrating overexpression of p53 by immunohistochemistry can be seen in the low-grade form.

Dr. Raffeld has also continued his studies on the role of the bcl-1 major breakpoint region in mantle cell lymphoma. These studies have shown that bcl-1 rearrangement and overexpression of the PRAD1 oncogene are specific to mantle cell lymphomas, and are not seen in other forms of B-cell malignancy. Moreover, PRAD1 overexpression can be shown in cases without detectable bcl-1 rearrangements by Southern blot.

Dr. Raffeld has also extended his observations on the molecular characterization of small non-cleaved cell lymphomas. Previously it had been shown that molecular differences exist between Burkitt's lymphoma and the non-Burkitt's variant. In the course of these studies, he identified a subgroup of Burkitt's lymphoma associated with mutations of the c-myc gene. These mutations are clustered in the second exon of the myc gene and may contribute to the pathogenesis of Burkitt's lymphoma.

Dr. Maryalice Stetler-Stevenson has been exploring the role of TIMP-1 and TIMP-2 (tissue inhibitors of metalloproteases) in Burkitt's lymphoma cell lines, as well as other cell lines. It was demonstrated that TIMP-1 is overexpressed in Burkitt's lymphoma cell lines and is associated with an increased proliferation rate *in vitro*. Moreover, TIMP-1 expression and secretion appears to be associated with an increase in tumorigenic and invasive behavior in the nude mouse model. Invasion of skin and nerve is seen only in TIMP-1 expressing lines but not in other transplanted lines. TIMP-1 expression appears to be an independent factor and is not associated with p53 mutations, EBV, or c-myc expression. As gelatinase expression may occur in these cells in response to the extracellular matrix, gelatinase A and gelatinase B were also studied but were found not to be associated with TIMP-1 expression in Burkitt cell lines.

Dr. Stetler-Stevenson has also been examining aneuploidy and cell cycle fraction analysis of benign and malignant tumors by DNA content distribution as determined by flow cytometry. In a series of gastrinomas from patients with Zollinger-Ellison syndrome, it was shown that patients with multiple stem cell aneuploidy had widespread disease, while all patients with single aneuploid populations had localized disease. High S-phase also correlated significantly with widespread disease ( $p=0.0039$ ). Therefore, DNA content analysis of gastrinomas provides important prognostic information that can be utilized in determining appropriate treatment of these patients.

Dr. Stetler-Stevenson is also using DNA content technology to analyze thyroid carcinomas from children and adults in the region contaminated with radiation from the Chernobyl accident. Data are being compared to those derived from adults in the United States with a history of radiation exposure, and American adults with no history of radiation exposure. Correlation between history of radiation exposure and the incidence of aneuploidy as well as S-phase in thyroid carcinomas is being analyzed. This information may also prove to be useful in the early detection of thyroid carcinoma in patients at high risk for this disease.

### Gene Regulation Section

The goal of the Section of Gene Regulation is to define the biochemical mechanisms employed during the transcription, processing and translation of RNA and to identify pathology resulting from aberrant regulation. Currently, the section has two main areas of research: 1) the transcriptional regulation of c-myc, and 2) the trans-activation of the gibbon ape leukemia virus by a set of factors binding to AP1 sites from T cells, some of which also interact with NFAT (nuclear factor of activated T cells).

The c-myc gene has multiple cis- and trans-elements both upstream and downstream of the major c-myc promoters P1 and P2. Three elements, originally described in the section, are being studied extensively. First, because cessation of c-myc transcriptional initiation has been shown to occur during pharmacologically induced differentiation of monomyelocytic leukemia cell lines and because this event appears to be a prerequisite for differentiation, experiments to identify a differentiation inducible repressor or a differentiation repressible activator were performed. Modulation of a factor as detected by loss of binding activity to a site 1500 bp upstream of promoter P1 was noted. The precise binding site was defined by deletional and mutational analysis. Functional transfection studies have indicated that this binding site serves as a positive element in undifferentiated leukemia cells. Following differentiation, the far upstream element, designated FUSE, ceases to stimulate c-myc expression. A 75 kD protein binding to the FUSE was purified and micro-sequence analysis allowed the cloning of a gene encoding the FUSE binding protein (FBP). The FBP protein possesses a novel structure including a new DNA binding motif. The FBP gene stimulates c-myc promoter mediated expression and deletion of the FUSE element diminishes this stimulation. Expression of FBP itself is regulated, being shut off during differentiation. Screening for genes related to FBP has revealed the existence of other highly related proteins suggesting that FBP may be the prototype for a new family of gene regulatory proteins.

Previously, a cis-element was identified in intron 1 of the human c-myc gene and was demonstrated to bind a nuclear protein. This element is mutated in most Burkitt's lymphomas. We have extended these investigations by identifying a 140 kD phosphoprotein responsible for this binding activity. Importantly, phosphorylation appears to be necessary for strong binding to the myc intron sequence. The 140 kDa protein has been purified from Hela cells

and the sequence of several internal tryptic peptides has been determined, revealing the myc-intron binding protein to be a new member of a known family of DNA binding proteins. Cloning of the full-length cDNA to allow *in vivo* and *in vitro* studies of protein structure and function are in progress. Additionally, a second protein component of the specific DNA-binding complex has been identified as a protein of approximately 35 kD. The role of this second factor in c-myc regulation is under investigation. Functional analysis of the binding for this complex is in progress. The site serves as a positive cis-element in HeLa cells.

One hundred bases upstream of the c-myc promoter is an element composed of multiple repeats of the sequence CCCTCCCA. This element stimulates expression from P2 and is essential for expression from P1. A complex array of factors interacts with site. A protein which binds to the CT-element in a highly sequence specific fashion was purified. Surprisingly, this factor also displays a marked preference for interacting with one of the two strands of the CT-element. Protein sequence analysis revealed this protein to be an isoform of hnRNP protein K; hnRNP protein K is a highly atypical hnRNP protein. It lacks any well described RNA binding motif and has no homology to any known RNA binding protein. Surprisingly, hnRNP protein K clearly possesses the same DNA binding motif present in FBP. Investigations are commencing to study the roles and interaction of FBP and hnRNP protein K as they effect c-myc expression.

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. Although this element contains an AP1 site, the factors which bind this site in T cells display properties distinct from those described for members of the fos/jun family.

Factors interacting with the enhancer of the gibbon-ape leukemia virus are important in T-cell activation. Studies of the GALV enhancer have led to the study of jun-d and associated factors in T-cell activation. The GALV binding complex has been shown to be composed of multiple components which pre-exist in resting T cells and are activated by a post-translational mechanism. jun-d seems to be the most important player of the AP1 family during the earliest stages of activation. Protein modification seems necessary for producing a functional jun-d protein; these changes include phosphorylation, but accumulating evidence suggests that other covalent changes are required as well.

A second component has been purified which binds cooperatively with jun-d protein showing a preference for natural over recombinant jun-d. This 23 kDa protein, designated "activator", stimulated jun-d binding more than one-hundred fold and stimulated GALV-AP1 site directed transcription, *in vitro*. Importantly, considerable evidence indicates that this same factor is part of

the NFAT (nuclear factor of activated T cells) complex. NFAT is a multi-component complex which plays a crucial role in directing the T-cell specific stimulation of IL-2 gene expression. Importantly, NFAT has been shown to contain an as yet undefined member(s) of the AP1 family, thus providing a unifying link for activator specificity. The activator displays novel properties which suggest that it should play an important role in T-cell function. Sufficient activator has been purified for protein sequencing and the section plans on obtaining a full-length cDNA as soon as suitable peptide sequence is available.

#### Office of the Chief

Dr. Susan Mackem is interested in elucidating the mechanisms at the molecular level by which pattern formation is regulated during embryonic development. Using limb morphogenesis as a model system for pattern formation that is readily amenable to various experimental manipulations, Dr. Mackem is employing subtractive hybridization approaches to isolate cDNA clones for genes that are induced during pattern formation in the embryonic limb and that play potential roles in determining limb-type identity. As a second, more directed approach, the role of known gene families thought to have regulatory functions in development is being investigated in the context of limb morphogenesis. Using such approaches, two novel non-Antennapedia homeobox genes with homeodomain sequences of some similarity to *Drosophila Abd-B (Ghox 4.7)* and *D11 (L5)* have been identified.

*Ghox 4.7* is expressed in a highly posteriorly restricted domain of the early limb bud correlating with the position of a functional zone regulating anterior-posterior (A-P) pattern of skeletal elements and also suggesting a role for this gene in patterning along the A-P axis. Preliminary results in transgenic mice confirm this impression, in that expression of *Ghox 4.7* in the anterior region of the developing limb results in a homeotic transformation of anterior limb skeletal elements to a posterior morphology.

The second gene (L5) has a highly restricted expression domain along the proximo-distal (P-D) axis of the limb, which changes with time as elements are progressively specified/determined along this axis. Expression is first seen early in the distal limb bud tip. At later times, the expression is localized more proximally, and is restricted to the region of the future anterior wrist or ankle. This type of expression is consistent with the known progressive determination of structures along the P-D axis in a proximal to distal sequence and suggests a role for L5 in the determination of positional identity, and hence pattern, along the P-D axis. Microsurgical manipulations (apical ridge excisions/grfts; retinoid treatment) are currently underway to analyze the expression pattern of this gene when the developmental program (pattern) is experimentally altered.

L5 is also expressed in the very early embryo; initially in Hensen's node at the onset of gastrulation and later in a temporal wave along the region of notochord adjacent to paraxial mesoderm that has not yet become



segmented into somites (receding caudally as development proceeds). The early L5 expression suggests roles in regulating formation of the primary embryonic axis during gastrulation and somitogenesis, which is being further investigated.

Long-term experiments to determine the function of these genes are currently underway, and include characterizing the effects of ectopic overexpression as well as ablation of expression of these genes using transgenic technology in mice, and avian retroviral expression vectors for transient expression experiments in chick embryos. Biochemical approaches are also being employed to identify the downstream "target" genes that are regulated by *Ghox 4.7* and L5 during limb morphogenesis.

Dr. Kathleen 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 have been completed on several clones, and selected clones are being studied in more detail. Several interesting functional classes of growth-regulated proteins have been revealed including a structurally unique class of nuclear tyrosine phosphatase, a novel GTP-binding protein associated with the cellular membranes, and a cell surface receptor with a large extracellular domain containing EGF repeat motifs and with seven transmembrane regions that couple signal transduction through G proteins. These proteins are being studied with regard to biochemical properties and potential physiological function.

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

PROJECT NUMBER

201 CB 00853-40 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. 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 STAFF YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOXES)

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

A

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

The Surgical Pathology Section provides expertise and diagnostic services in the field of Anatomic Pathology for the Institutes and Clinical Center patients, and collaborates with the research staff in those investigations which involve the use and study of human pathological material. Approximately 6,000 surgical specimens and biopsies (more than 60,000 slides which include routine and a variety of special stains) were accessioned last year. These include more than 2,000 fresh human tissues. A tissue procurement nurse works in close collaboration with the surgical pathology staff and helps in the distribution of tissues to scientists throughout the NIH.

The members of the section also participate in a variety of teaching and interdepartmental conferences (medicine branch, surgery branch, etc.) in which patient diagnosis and modalities of therapy are discussed, assisting in this way, to provide better patient care. Other objectives of the Surgical Pathology section include, to carry independent research by the members of the section, and to provide a residency program in anatomic pathology.

The section also provides consultant services to the community as well as to pathologists throughout the country.

Other Professional Personnel:

I. Lubensky	Expert	LP NCI
G. Jaffe	Expert	LP NCI
J. Taubenberger	Expert	LP NCI
D. Kleiner	Medical Staff Fellow	LP NCI
+H. Hollingsworth	Medical Staff Fellow	LP NCI
+C. Moskaluk	Medical Staff Fellow	LP NCI
+T. Giordano	Medical Staff Fellow	LP NCI
+S. Barksdale	Medical Staff Fellow	LP NCI
+M. Roth	Medical Staff Fellow	LP NCI
+M. Buck	Medical Staff Fellow	LP NCI
+C. Phillips	Medical Staff Fellow	LP NCI
+B. Cheshire	Medical Staff Fellow	LP NCI
+Z. Zhuang	Medical Staff Fellow	LP NCI
*J. Stern	Consultant in Dermatopathology	LP NCI
D. Katz	Consultant in Neuropathology	LP NCI

Objectives:

- (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.

Histologic 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 CC49 to treat breast cancer; 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.

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+These physicians are full-time Residents in the Laboratory of Pathology.

\*This Associate Pathologist spends part time in the activities of the Surgical Pathology Section.

Publications:

Campo E, Brunier MN, Merino MJ. Small cell carcinoma of the endometrium with associated ocular paraneoplastic syndrome. *Cancer* 1992;69:2283-8.

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Straus K, Lippman M, Danforth D, Swain S, Cowan K, MacDonald H, d'Angelo T, Merino MJ, Bader J, Rosenberg S, Glatstein E. Results of the NCI early breast cancer trial. *J Natl Cancer Inst* 1992;11:27-32.

Fernandez PL, Merino MJ, Nogales FF, Charonis AS, Stetler-Stevenson WG, Liotta LA. Immunohistochemical profile of basement membrane proteins and 72 kilodalton type IV collagenase in the implantation placental site: An integrated view. *Lab Invest* 1992;66:572-9.

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Jensen JC, Pogrebniak HW, Pass HI, Buresh C, Merino MJ, Kauffman D, Venzon D, Langstein HN, Norton JA. Role of tumor necrosis factor in oxygen toxicity. *J Appl Physiol* 1992;72:1902-7.

Taubenberger JK, Merino MJ, Medeiros LJ. A thyroid biopsy with histologic features of both Riedel's thyroiditis and the fibrosing variant of Hashimoto's thyroiditis. *Hum Pathol* 1992;23:1072-5.

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Pogrebniak HW, Merino MJ, Hahn SM, Mitchell JB, Pass HI. Spin trap salvage from endotoxemia: The role of cytokine down-regulation. *Surgery* 1992;112:130-9.

Alexander HR, Doherty GM, Venzon DJ, Merino MJ, Fraker DL, Norton JA. Recombinant interleukin-1 receptor antagonist (IL-1ra): effective therapy against gram-negative sepsis in rats. *Surgery* 1992;112:188-94.

Lange JR, Alexander HR, Merino MJ, Doherty GM, Norton JA. Interleukin-1 $\alpha$  prevention of the lethality of *Escherichia coli* peritonitis. *J Surg Res.* 1992;52:555-9.

Lakshmanan M, Reynolds JC, Del Vecchio S, Merino MJ, Norton JA, Robbins J. Pelvic radioiodine uptake in a rectal wall teratoma after thyroidectomy for papillary carcinoma. *J Nucl Med* 1992;33:1848-50.

Campo E, Merino MJ, Liotta L, Neumann R, Stetler-Stevenson WG. Distribution of the 72 kDa type IV collagenase in non-neoplastic and neoplastic thyroid tissue. Hum Pathol 1992;23:1395-1401.

Barth R, Merino MJ, Solomon D, Yang J, Baker A. A prospective study of the value of Tru-Cut needle biopsy and fine needle aspiration in the diagnosis of soft tissue masses. Ann Surg 1992;112:536-43.

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

PROJECT NUMBER

Z01 CB 09145-09 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Katz Neuropathologist OCD NINDS

COOPERATING UNITS (if any)

Surgical Pathology Section, 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 STAFF YEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOXES!

- (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, NINDS. 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 variety of clinicopathologic investigations.

Major Findings:

The neuropathology service continues to function: (1) to provide a specialized diagnostic service for neuropathological surgical 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: The brain is examined in approximately 90% of all NIH autopsies (approximately 90 brains/year) and one-half of these are primarily neurological cases. A significant proportion of the remainder exhibit neuropathological findings as well. Current patient care material includes dementia and other degenerative neurological diseases, AIDS (predominantly pediatric), primary brain tumors, and systemic cancer. 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 350 neurosurgical specimens are examined yearly, including both submitted and in-house cases. Approximately 35 intra-operative frozen section consultations are provided yearly. Current case material includes primary brain tumors, pituitary adenomas, metastatic tumors, electrocorticographically-guided resections for temporal lobe seizures, hemangioblastomas, and muscle biopsies.

Conferences: The case material described above is also utilized for resident teaching conferences and neurology conferences, including presentations at NINDS 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, NINDS); vasculopathy and white matter degeneration in the elderly (NIA).

2. Pituitary adenomas: correlative study of adenomas, particularly in Cushing's disease (NICHD, NINDS).

3. AIDS (pediatric): correlative diagnosis of AIDS encephalopathy.

4. Multiple sclerosis: correlation of acute lesions with neuroimaging; study of non-MS lesions mimicking MS on MRI.

5. Neuroimaging: *in vitro* NMR characteristics of normal brain tissue at autopsy.
6. HTLV-1-associated myelopathy: clinicopathologic study, correlation with PCR.
7. Experimental treatment of malignant brain tumors: gene therapy, immunotoxin (NINDS); pediatric tumors (NCI).

Publications:

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Smith M, Katz DA, Harris JO, Frank JA, Kufta CV, McFarlin DE: Systemic histiocytosis presenting as multiple sclerosis. *Ann Neurol* 1993;33:549-54.

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

PROJECT NUMBER

Z01 CB 09193-04 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Malignant Changes Associated with Sclerosing Adhesions

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

PI: M. Merino Chief, Surgical Pathology Section LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOXES)

(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.)

Sclerosing adenosis is a proliferative breast lesion with pseudoinvasive features frequently misdiagnosed as infiltrating carcinoma. The purpose of this study will be: 1) evaluate the premalignant potential of this lesion; 2) its association with carcinoma and 3) evaluate the integrity of the basement membrane. Patients with sclerosing adenosis in which *in situ* cancers develop are probably at a much higher risk to evolve to an invasive cancer.

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

PROJECT NUMBER

Z01 CB 09361-03 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

P-Glycoprotein Expression in Breast Cancer

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

PI:	M. Merino	Chief, Surgical Pathology Section	LP NCI
OTHER:	V. Monterroso	Visiting Fellow	LP NCI
	K. Cowan	Chief, Medical Breast Cancer Section	MB NCI
	J. O'Shaughnessy	Senior Staff Fellow	COP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A

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

The multidrug-resistance (MDR) gene product, P-glycoprotein (P170) has been known to be increased in tumors resistant to chemotherapeutic drugs. We will evaluate the presence of P-glycoprotein in cases of breast cancer, utilizing immunohistochemical techniques. Biopsies obtained before and after treatment will be evaluated, utilizing commercial antibodies.

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

PROJECT NUMBER

Z01 CB 09362-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Phase I Study of IV <sup>177</sup>Lu Murine CC49 Patients with Breast Cancer

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

PI:	J. O'Shaughnessy	Senior Staff Fellow	COP NCI
OTHER:	K. Cowan	Chief, Medical Breast Cancer Section	MB NCI
	J. Schlom	Chief, Immunology Section	LTIB NCI
	M. Merino	Chief, Surgical Pathology Section	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOXES)

- (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 purposes of this study are:

- 1) to determine the presence of the monoclonal antibody CC49 in tissues;
- 2) to study the toxicity of <sup>177</sup>Lu CC49 in patients with advanced adeno-carcinoma
- 3) to study the ability of CC49 to image known metastases

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

PROJECT NUMBER

Z01 CB 09380-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Histological Findings in Premenopausal Women with Breast Cancer

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

PI:	M. Merino	Chief, Surgical Pathology Section	LP NCI
OTHER:	P. Bertheau	Visiting Fellow	LP NCI
	J. O'Shaughnessy	Senior Staff Fellow	COP NCI

COOPERATING UNITS (if any)

LABORATORY

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOXES)

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

A

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

Histological evaluation of biopsies in premenopausal patients with breast cancer will be done: 1) in search of precancerous lesions; 2) to determine type and extension of tumor and 3) to evaluate histologic factors that may predict outcome.

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

PROJECT NUMBER

Z01 CB 09381-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Evaluation of Prognostic Tumor Markers in Premenopausal Patients with Breast CA

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

PI:	M. Merino	Chief, Surgical Pathology Section	LP NCI
OTHER:	P. Bertheau	Visiting Fellow	LP NCI
	P. Steeg	Research Biologist	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2

PROFESSIONAL:

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.)

Prognostic markers such as cerb-2, P53, nm23 and TGF- $\beta$  will be studied by immunohistochemistry and *in situ* hybridization in order to determine their role in prognosis of young women with breast cancer.

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

PROJECT NUMBER

Z01 CB 09166-06 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Travis Chief, Pulmonary Pathology Section LP NCI  
 OTHER: V. Ferrans Chief, Ultrastructure Section IR PA NHLBI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI

LABORATORY

Laboratory of Pathology

SECTION

Pulmonary Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF 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 80 patients with idiopathic pulmonary fibrosis have already been reviewed and the data are currently being analyzed. Biopsies from 48 patients with pulmonary histiocytosis X are also being written up. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings.

Lung specimens from 26 patients with Churg-Strauss syndrome have been collected and are being analyzed for publication.

Publications:

Travis WD, Fox CH, Devaney KO, Weiss LM, O'Leary TJ, Ognibene F, Suffredini A, Rosen M, Cohen MB, Shelhamer J. Lymphoid pneumonitis in 50 adult HIV-infected patients: Lymphocytic interstitial pneumonitis versus nonspecific interstitial pneumonitis. *Hum Pathol* 1992;23:529-41.

Hoffman GS, Kerr GS, Leavitt RY, Hallahan CW, Lebovics RS, Travis WD, Rottem M, Fauci AS. Wegener's granulomatosis: a prospective analysis of 158 patients. *Ann Int Med* 1992;116:488-98.

Lebovics RS, Hoffman GS, Leavitt RY, Kerr GS, Travis WD, Kammerer W, Hallahan C, Fauci AS. The management of subglottic stenosis in patients with Wegener's granulomatosis. *Laryngoscope* 1992;102:1341-5.

Travis WD. Pathologic features. Chapter 9. In: Walzer P, ed. *Pneumocystis carinii* pneumonia. New York: Marcel Dekker, Inc., 1993;155-80.

Travis WD, Hoffman GS, Leavitt RY, Lebovics RS. Problems in interpretation of head and neck biopsies for Wegener's granulomatosis. In: Tanabe T, Yoshiki T, Okayasu T, eds. *Intractable vasculitis syndromes*. Sapporo, Japan: Hokkaido University Press, 1993;27-52.

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

PROJECT NUMBER

Z01 CB 09365-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Pulmonary Pathology

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

PI: W. Travis Chief, Pulmonary Pathology Section LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Pulmonary Pathology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3

PROFESSIONAL:

2

OTHER:

1

CHECK APPROPRIATE BOXES)

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

A

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

The section of Pulmonary Pathology, together with the Cytopathology Section, Hematopathology Section, Postmortem 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 pulmonary section provides diagnostic services for several pulmonary groups at the NIH including the NIAID (Dr. Fauci) for vasculitis, the NHLBI, Pulmonary Branch (Dr. Crystal) for interstitial lung disease, Critical Care (Dr. Masur) for lung disease in immunocompromised patients; and the NCI, Surgery Branch (Dr. Pass) for lung cancer and malignant mesothelioma protocols. The pathology for all interstitial lung disease patients with lung biopsies is presented at Dr. Crystal's weekly, Friday noon conference. Surgical and autopsy lung specimens are reviewed on a daily basis for these various pulmonary groups.

The pulmonary material is being utilized for research purposes to study lung cancer, malignant mesothelioma, and nonneoplastic interstitial lung disease. Electron microscopic and immunohistochemical techniques are being utilized to study these diseases. A prospective collaborative project has been undertaken in conjunction with Dr. Lance A. Liotta, Dr. Curtis C. Harris, and the lung cancer group at Mayo Clinic to investigate the molecular biology of lung cancer.

In addition, a collaborative working relationship has been set up with the Pulmonary and Mediastinal Branch at the Armed Forces Institute of Pathology. This has allowed study of a wide variety of rare and unusual lung diseases including neuroendocrine tumors of the lung, pulmonary epithelioid hemangioendotheliomas, sclerosing hemangiomas, primitive neuroectodermal tumors presenting in the lung, and sarcomatoid carcinomas of the lung.



Objectives:

The objectives of the Pulmonary Section are: (a) to provide diagnostic services in pulmonary pathology for the clinical research protocols conducted at NIH. This includes generating pathology reports for current cases and presenting the pathologic findings of 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, especially pulmonary pathology; (d) to collaborate with investigators in research involving the use and study of human materials; (e) to establish an internationally recognized pulmonary pathology training program for pathology residents in collaboration with the Armed Forces Institute of Pathology.

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 pulmonary pathology material.

Two major projects are currently in progress on the study of lung cancer:

1) A prospective collaborative project in conjunction with Dr. Lance A. Liotta, Dr. Curtis C. Harris, and the lung cancer group at Mayo Clinic to investigate the molecular biology of lung cancer. So far, tissue samples of lung tumor and nontumorous lung, as well as blood samples have been collected on 100 lung cancer patients. Each of these patients has completed a comprehensive epidemiologic questionnaire and has detailed information available regarding clinical history. Followup will become available through the Mayo Clinic Department of Thoracic Disease. Molecular studies are underway in Dr. Harris' and Dr. Liotta's laboratories on these samples.

2) A retrospective study of 100 patients with neuroendocrine tumors from the AFIP files has been undertaken. The histologic features of these cases has been characterized and followup is being completed. This will provide important information about the clinical behavior of large cell neuroendocrine carcinoma, a tumor recently described by us at NCI. A prospective collaborative project has been undertaken in conjunction with Dr. Lance A. Liotta, Dr. Curtic C. Harris, and the lung cancer group at Mayo Clinic to investigate the molecular biology of lung cancer.

Publications:

Feuerstein IM, Jicha DL, Pass HI, Chow CK, Chang R, Ling A, Hill SC, Dwyer AJ, Travis WD, Horowitz ME, Steinberg SM, Frank JA, Doppman JL. Pulmonary metastases: MR imaging with surgical correlation--a prospective study. *Radiology* 1992;182:123-9.

Broers JLV, Jensen SM, Travis WD, Pass H, Whitsett JA, Singh G, Katyal SL, Gazdar A, Minna JD, Linnoila RI. Expression of SP-A and clara cell 10 kD mRNA in neoplastic and non-neoplastic human lung tissue as detected by *in situ* hybridization. *Lab Invest* 1992;66:337-46.

Mulé, JJ, Custer MC, Travis WD, Rosenberg SA. Cellular mechanisms of the antitumor activity of recombinant interleukin-6 in mice. *J Immunol* 1992;148:2622-9.

Koss MN, Travis W, Moran C, Hochholzer L. Pseudomesotheliomatous adenocarcinoma: a reappraisal. *Semin Diag Pathol* 1992;9:117-23.

Tsubota YT, Kawaguchi T, Hono T, Nishino E, Travis WD. A combined small cell and spindle cell carcinoma of the lung: Report of a unique case with immunohistochemical and ultrastructural studies. *Am J Surg Pathol* 1992;16:1108-15.

Filling-Katz MR, Miller S, Merrick HF, Travis W, Gregg RE, Tsokos M, Comly M, Mackie S, Lebovics R, Brady RO, Pentchev PG. Clinical, pathological and biochemical features of a cholesterol lipidosis accompanied by hyperlipidemia and xanthomas. *Neurology* 1992;42:1768-74.

Atkinson JC, Travis WD, Slocum L, Ebbs WL, Fox PC. Serum anti-SS-B/La and IgA rheumatoid factor are markers of salivary gland disease activity in primary Sjögren's syndrome. *Arthritis Rheum* 1992;35:1368-72.

Moran CA, Hochholzer L, Fishback N, Travis WD, Koss MN. Mucinous (so-called colloid) carcinomas of the lung. *Mod Pathol* 1992;5:634-8.

Kragel PJ, Luibel FJ, Travis WD. Sarcomatoid renal carcinoma with an angiosarcomatoid component: light microscopic and immunohistochemical study. *Urology* 1992;40:381-4.

Travis WD, Li CY. Mast cell disease. In: Kaliner MA, Metcalfe DD, eds. *The role of the mast cell in health in disease*, vol. 62, chapter 30. New York: Marcel Dekker, Inc., 1993;723-41.

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

PROJECT NUMBER

Z01 CB 09364-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Postmortem Pathology

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

PI: G. Jaffe Chief, Postmortem Pathology Section LP NCI

OTHER: D. Kleiner, C. Baker, P. Howley, D. Levens, L. Liotta,  
 S. Mackem, T. O'Leary, A. Larner, A. Ginsberg, J. Taubenberger,  
 H. Hollingsworth, K. Gardner, S. Barksdale, C. Moskaluk,  
 T. Giordano, M. Buck, M. Roth, L. Cheshire, J. Teruya C. Phillips,  
 Z. Zhuang, W. Stetler-Stevenson, I. Lubensky, L. Abruzzo, M. Jerome,  
 A. Dock, J. Rainey, W. Roberts, D. Katz

COOPERATING UNITS (if any)

LABORATORY

Laboratory of Pathology

SECTION

Postmortem Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

24

PROFESSIONAL:

21

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 Postmortem Pathology Section, along with the Cytopathology Section, Hematopathology Section, Surgical Pathology Section, and Ultrastructural Pathology Section, provide a complete service in Anatomic Pathology for the Clinical Center as well as other institute patients. In addition, when the use and study of human pathological material is requested by research staff of any of the institutes, the Postmortem Section makes every effort to collaborate with and/or supply the researchers with the human tissues upon approved request.

The autopsy material is utilized by staff and residents for research projects involving clinicopathological correlation and characterization of disease processes. Currently, several projects are on-going: clinical-pathological studies in dementia; MRI correlations with normal tissue and demyelinating disease (multiple sclerosis); experimental therapy of malignant brain tumors (both primary and metastatic); study involving chronic granulomatous disease; evaluation of fungal infections in immunocompromised hosts; preliminary work evaluating cellular adhesion molecules involving organs in interleukin-2 treated individuals; normal tissues used for purification of antigens to make antibodies and titration of new antibodies using autopsy material.

Additionally, a database of major autopsy diagnoses from 1982 through 1990 is being compiled, with pertinent historical information included.

Objectives:

1. To provide diagnostic services in autopsy pathology and to generate final anatomic (autopsy) diagnoses for the clinical records.
2. To provide a residency program in anatomic pathology.
3. To collaborate with investigators in research involving human tissues from autopsy material.
4. To carry out independent research.

The proposed research program includes (a) continuing to provide the services described; (b) increasing interaction with clinical branches in the design and the evaluation of protocols; (c) providing opportunities for residents to participate in teaching and research projects; (d) developing data retrieval systems for the autopsy material.

The basic setting in which this occurs (autopsy suite) has been specially equipped for safety, which 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 used to protect our staff and residents from exposure to contaminated tissues with high risk infectious agents.

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

PROJECT NUMBER

Z01 CB 00852-40 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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
OTHER:	A. Abati	Deputy Chief, Cytopathology Section	LP NCI
	Y. Hijazi	Expert	LP NCI
	D. Gagneten	Fellow	LP NCI
	C. Copeland	Cytotechnologist	LP NCI
	L. Galito	Biologist	LP NCI
	A. Wilder	Cytotechnologist	LP NCI
	E. Sanders	Bio. Lab. Technologist	LP NCI
	P. Fetsch	Medical Technologist	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
8	2.5	5.5

CHECK APPROPRIATE BOXES)

(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 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 and immunocytochemistry. In conjunction with Dr. M.A. Stetler-Stevenson in the Hematopathology Section, we are initiating flow cytometry as an ancillary diagnostic technique in bladder washings.

The fine needle aspiration service is designed to afford maximal flexibility for clinicians and patients. Clinicians may request that: 1) a pathologist perform the aspiration; 2) a cytotechnologist assist the clinician in handling the specimen; 3) aspirations of deep lesions be performed by the radiologist with the assistance of a cytotechnologist to evaluate adequacy of the specimen.

An example of one collaborative clinical research project involves clinical trials currently being conducted to study the use of the monoclonal antibody RFB4-RICIN A chain conjugate for refractory CD22 positive B-cell lymphoma. Our collaborative effort in this project involves the cytomorphologic evaluation of cytopathology specimens in order to: 1) document the presence of lymphoma and CD22 positivity prior to initiation of immunotoxin therapy and 2) monitor response by evaluating CD22 levels and presence of immunotoxin.

Another collaborative project with the Whitman-Walker AIDS clinic is evaluating the prevalence of cervical premalignant lesions in HIV infected women. A few reports in the literature have cited a high rate of dysplasias in HIV infected women. These findings, if substantiated, have implications for cervical screening recommendations for this population.

Two previous projects have included evaluation of the comparative utility of core needle biopsy and fine needle aspiration in the diagnosis of soft tissue lesions, and the role of cytology in the diagnosis of *Pneumocystis* in HIV infected patients.

Major Findings:

Approximately 3500 cytology specimens were evaluated over the past year in the Cytopathology Section. Diagnoses are generally available within 24 hours of receipt. Preliminary diagnoses on STAT cases are communicated within 1-2 hours. Cytology is no longer simply a screening modality: Cytologic evaluation often provides definitive diagnoses which dictate patient care and treatment.

Fine needle aspiration specimens have continued to increase by 10-20% per year. 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 50% to 160 cases.

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:

Barth RJ, Merino MJ, Solomon D, Yang FC, Baker AR. A prospective study of the value of core needle biopsy and fine needle aspiration in the diagnosis of soft tissue masses. *Surgery* 1992;112:536-43.

Levine SJ, Kennedy D, Shelhamer JH, Kovacs A, Feuerstein IM, Gill VJ, Stock F, Solomon D, Boylen CT, Masur H, Ognibene FP. Diagnosis of *Pneumocystis carinii* pneumonia by multiple lobe site-directed bronchoalveolar lavage with immunofluorescent monoclonal antibody staining in HIV-infected patients receiving aerosolized pentamidine chemoprophylaxis. *Am Rev Respir Dis* 1992;146:838-43.

Solomon D. Fine needle aspiration of the thyroid. *Thyroid Today* (in press)

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

PROJECT NUMBER

Z01 CB 00897-10 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

**Immunocytochemistry as an Adjunct to Cytopathological Diagnosis**

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:	P. Fetsch	Medical Technologist	LP NCI
	Y. Hijazi	Expert	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

.5

OTHER:

.5

CHECK APPROPRIATE BOXES:

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

A

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

Immunocytochemistry is proving to be a valuable diagnostic adjunct to cytopathologic diagnosis. We are evaluating the utility of a variety of polyclonal and monoclonal antibodies in different diagnostic settings.

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 CD5, CD3, CD4 and CD8. 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 lymphomas.

Another project utilizing immunocytochemistry as an adjunct to routine light microscopic cytologic diagnosis, involves distinguishing polyoma viral effects from atypia secondary to cyclophosphamide therapy in urine specimens. A large population of patients followed at the NIH are receiving cyclophosphamide therapy on an on-going basis for the treatment of both benign and malignant disease. Cytomorphologic abnormalities have been described in the urine of cyclophosphamide-treated patients and have been confused cytologically with urinary tract neoplasia, the incidence of which is also increased following cyclophosphamide therapy. Furthermore, the cytologic features of polyoma virus cytopathic effect in the urine also overlap the features of cyclophosphamide effect and neoplasia. We have used immunocytochemistry with a polyclonal antibody to polyoma virus to document the presence of virus in the urine specimens of some patients in order to better define the distinguishing characteristics of cyclophosphamide effect, neoplasia and polyoma virus.

A collaborative project with endocrinology is evaluating the utility of FNA in the workup of submucosal intestinal nodules in patients with Zollinger Ellison syndrome (ZES). FNA appears to be a sensitive diagnostic technique in this setting. Immunocytochemistry is being used to confirm neuroendocrine differentiation of tumor cells.

Major Findings:Immunocytochemistry in the evaluation of lymphoid cell populations:

We have investigated 630 specimens, including 270 pleural and peritoneal effusions, 127 cerebrospinal fluids, and 217 fine needle aspiration specimens. We have found 310 cases to be positive for lymphoma and 192 to be reactive in nature. Of the 310 positive cases, 260 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 44 cases on the basis of aberrant marker phenotype or TdT positivity. Acute nonlymphocytic leukemia was diagnosed in three 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. Particularly in the setting of HIV-associated lymphoma, unusual sites of initial presentation and/or the debilitated condition of many patients may preclude more invasive tissue biopsy diagnostic techniques. In these cases, a definitive cytopathologic diagnosis obviates the need for more invasive diagnostic procedures.

In the investigation of the utility of cytology in ZES, FNA was performed in 14 ZES patients with submucosal nodules. Of a total of 21 aspirated nodules, 11/12 were identified as neuroendocrine and 8/9 as nonneuroendocrine. FNA has been more sensitive in diagnosis than jumbo forceps biopsy and snare polypectomy.

Publications:

Benya RV, Metz DC, Hijazi YM, Fishbeyn VA, Piseigna JR, Jensen RT. Fine needle aspiration cytology of submucosal nodules in patients with Zollinger-Ellison syndrome. Am J Gastroent 1992;88:258-65.



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

PROJECT NUMBER

Z01 CB 09153-07 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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	Surgery Branch	SB NCI
	S. Rosenberg	Chief, Surgery Branch	SB NCI
	J. Yannelli	Surgery Branch	SB NCI
	P. Fetsch	Medical Technologist	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 STAFF YEARS:

0.3

PROFESSIONAL:

.15

OTHER:

.15

CHECK APPROPRIATE BOXES)

- (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 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:

Over 260 tumor suspensions have been evaluated for tumor associated antigens and for phenotypic analysis of tumor infiltrating lymphocytes including: melanomas, renal cell carcinomas and sarcomas.

Over 420 tumor infiltrating lymphocyte (TIL) cultures have been examined. Cytologically, TIL cultures consist of a monomorphic population of activated lymphoid cells resembling an immunoblastic lymphoma. The majority of reactive lymphoid cells from TIL cultures are CD3 positive with a variable proportion of cells positive for CD4 or CD8. In less than 2% of cultures, rare residual tumor cells are identified.

Publications:

Schwartzentruber DJ, Solomon D, Rosenberg SA, Topalian SL. Characterization of lymphocytes infiltrating human breast cancer: Specific immune reactivity can be detected by measuring cytokine secretion. J Immunotherapy 1992;12:1-12.

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

PROJECT NUMBER

Z01 CB 09176-05 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

COOPERATING UNITS (if any)

DCPC: CDC

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

.25

PROFESSIONAL:

.25

OTHER:

CHECK APPROPRIATE BOXES)

- (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.)

As Chief of Cytopathology, I have participated in numerous activities to broaden and improve cervical cancer Pap smear screening, including: 1) the development and refinement of The Bethesda System (a uniform descriptive diagnostic terminology for Pap smear reporting); 2) organizing and co-chairing "50 Years of Pap Screening: Milestones and Missions", a national conference held in October 1992 to develop recommendations for improving cervical cancer screening; 3) drafting of "The National Strategic Plan for Breast and Cervical Cancer - Cervical Cancer Quality Assurance"; 4) development of "Interim Guidelines for Management of Abnormal Cervical Cytology".

Major Findings:

1) The 1988 Bethesda System has had a significant impact on the practice of gynecologic cytopathology. A survey, conducted in early 1991 by the College of American Pathologists, revealed that 87% of the labs surveyed had already implemented TBS, or were planning to do so in the near future. A "Second Conference" on The Bethesda System, held April 29 and 30, 1991, provided open exchange of data, lively debate and a forum for critical analysis of TBS. The revised TBS has been significantly streamlined and simplified. As a member of the Criteria Committee, I am contributing to a TBS reference atlas which will include morphologic criteria and accompanying photomicrographs.

2) The national conference "50 Years of Pap Screening" brought together recognized leaders in the fields of public health, womens' advocacy, gynecology, pathology, and primary care, to discuss and suggest how cervical cancer screening should be improved. The summary of recommendations, which resulted from the meeting, is in preparation.

3) The National Strategic Plan for Breast & Cervical cancer, prepared under the aegis of the NCI, CDC and FDA, outlines needs and actions which form the basis for a coordinated approach to early detection of these cancers. The plan is aimed at achieving or surpassing the Healthy People 2000 objectives pertaining to breast & cervical cancers.

4) The Interim Guidelines for Management of Abnormal Cytology, represents a collaborative effort on behalf of the American College of Obstetricians and Gynecologists, and NCI. Currently in the United States, there is no consensus as to the management of low grade cervical lesions; these guidelines will outline therapeutic options for patient management. This is intended to be an interim measure until large-scale clinical trials provide additional data upon which to base recommendations.

In addition, I have been involved as a cytopathology resource person in numerous meetings and working groups including: The College of American Pathologist's Cytopathology Committee; Executive committee of the American Society of Cytology; several CDC workshops on quality assurance in breast and cervical cancer; a CDC expert panel on cervical disease in HIV-infected women; and a videodisc collaboration with the National Library of Medicine on cervical cancer.

Publications:

Solomon D. Nomenclature for cervicovaginal cytology. In: Wied GL, Keebler CM, Koss LG, Patten SF, Rosenthal DL, eds. Compendium on diagnostic cytology. Tutorials of Cytology, Chicago, 1992.

NCI Workshop: The 1991 Bethesda System for Reporting Cervical/Vaginal Cytologic Diagnoses. Acta Cytol 1993;37:115-24.

Solomon D, Sedlacek TB. Problems in the cervical cancer screening process. The Female Patient 1993;18:64-6.

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

PROJECT NUMBER

Z01 CR 09363-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Glutathione S-transferase-pi and P-glycoprotein in FNA of Breast Tumors

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

PI:	K. Cowan	Senior Investigator	COP DCT NCI
OTHER:	D. Solomon	Chief, Cytopathology Section	LP NCI
	J. O' Shaughnessy	Medical Officer	COP DCT NCI
	T. Tolcher	Medical Officer	COP DCT NCI
	P. Fetsch	Medical Technologist	LP NCI

COOPERATING UNITS (if any)

DCT LP

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

.20

PROFESSIONAL:

.10

OTHER:

.10

CHECK APPROPRIATE BOX(ES)

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

A

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

The glutathione S-transferases are a group of enzymes that have been shown to be active in the detoxification of certain drugs, carcinogens and metabolites. Increased expression of the placental form of the enzyme has been found in association with carcinogenesis and drug resistance. Some investigators have found increased expression of the enzyme, measured by RNA slot blot and protein assays, to be inversely related to estrogen receptor positivity in malignant breast tumors.

We are prospectively studying fine needle aspirates of breast cancer patients to assess glutathione S-transferase and expression of a drug resistance marker, P-glycoprotein, before and after chemotherapy to determine if a relationship exists between levels of expression and response to therapy. Thus far, we have evaluated 35 patient aspirate samples.

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

PROJECT NUMBER

Z01 CB 09383-01 LP

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 HLA Expression in Melanoma Before and After Interferon

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

PI:	D. S. Rosenberg	Chief, Surgery Branch	SB NCI
OTHER:	F. Marincola	Surgery Fellow	SB NCI
	A. Abati	Deputy Chief, Cytopathology Section	LP NCI
	P. Fetsch	Medical Technologist	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH  
 Laboratory of Pathology

SECTION  
 Cytopathology Section

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:  
 .35

PROFESSIONAL:  
 .10

OTHER:  
 .25

CHECK APPROPRIATE BOXES!

- (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.)

HLA Class I expression on tumor cells is involved in the anti-tumor immune response. Using known melanoma cell lines, we assessed expression of the A and B loci on cell membranes using monoclonal antibodies before and after treating the cells with interferon. We concluded that the A locus is expressed in melanoma cultured cell lines and is not up-regulated with interferon  $\gamma$ . The B locus is frequently down regulated but is sensitive to interferon  $\gamma$  up-regulation. We are now using these same techniques on melanoma patients by performing fine needle aspirations of superficial melanoma lesions before and after treatment with interferon  $\gamma$  or interleukin 2.

Publications:

Marincola F, Shamamiam P, Simonis T, Abati A, Fetsch P, Hacket J, O'Dea T, Rosenberg S. Locus specific analysis of HLA class I expression in melanoma cell lines. Cancer Res (in press)

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

PROJECT NUMBER

Z01 CB 09187-04 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Transforming Growth Factor (TGF)- $\beta$  in the Differentiation of Neuroblastoma

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

PI: M. Tsokos Chief, Ultrastructural Pathology LP NCI  
 Section  
 OTHER: J. Keleti Visiting Scientist LC NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1

PROFESSIONAL:

4/4

OTHER:

CHECK APPROPRIATE BOXES)

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

D

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

In a recent immunohistochemical study we found lack of expression of TGF- $\beta$ 1, 2, and 3 by neuroblastoma cells, except for well differentiated ganglion cells in ganglioneuroblastomas and in normal ganglia. These data suggested a possible role of TGF- $\beta$  in the differentiation of human neuroblastoma. This hypothesis will be further pursued in the following ways: (1) TGF- $\beta$  expression and secretion will be studied in neuroblastoma cell lines before and after differentiation with known differentiating agents, such as retinoic acid and TPA. (2) Direct effects of exogenously added TGF- $\beta$  in undifferentiated and differentiated neuroblastoma cell cultures will be determined. (3) Blocking antibodies and antisense TGF- $\beta$  oligonucleotides will be used to modulate possible actions of TGF- $\beta$  on neuroblastoma cells *in vitro*. Preliminary data have shown induction of TGF- $\beta$ 1 mRNA in SH-SY5Y neuroblastoma cells following treatment of cultures with TPA or retinoic acid, both of which promoted differentiation. In contrast, another neuroblastoma cell line (IMR-32) which showed minimal morphologic differentiation with the same agents, showed only mild increase in TGF- $\beta$ 1 mRNA levels. The differences in the TGF- $\beta$  expression by neuroblastoma cells before and after differentiation may be the result of induction of surface receptors by the differentiation agents and secondary synthesis of TGF- $\beta$ , a phenomenon which will be further pursued by binding studies to determine possible changes of TGF- $\beta$  receptors on the surface of neuroblastoma cells before and after treatment with differentiating agents.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 09354-03 LP
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Role of Transforming Growth Factor (TGF)- $\beta$ in Rhabdomyosarcoma		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	M. Tsokos Chief, Ultrastructural Pathology Section	LP NCI
OTHER:	J. Keleti Visiting Fogarty Fellow	LP NCI
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Ultrastructural Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS: 1	PROFESSIONAL: 3/4	OTHER: 1/4
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 <input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) TGF- $\beta$ 1 has an inhibitory effect in normal myogenesis. It also causes reversible inhibition of fusion and expression of muscle specific genes by skeletal and smooth muscle myoblasts <i>in vitro</i> without affecting cell proliferation. Therefore, it has been postulated that TGF- $\beta$ 1 may have a role in muscle regeneration and may prevent precocious fusion of embryonic myoblasts. The role of TGF- $\beta$ in myogenous tumors is unknown. Using the avidin-biotin immunoperoxidase technique, we found that rhabdomyosarcomas (RMS) stain intensely for TGF- $\beta$ 1 and TGF- $\beta$ 3, but not TGF- $\beta$ 2 <i>in vivo</i> . This project aims at investigating the possible role of TGF- $\beta$ in the growth and differentiation of RMS <i>in vitro</i> . Three established RMS cell lines (RD, Birch and RH18) are selected for the studies. Using the 3H-thymidine incorporation assay, we found that TGF- $\beta$ 1 at concentrations of 0.25 to 1 ng/ml inhibits cell growth in all 3 RMS cell lines grown in serum-free media. This inhibitory effect appeared to be specific to exogenous, but not endogenous TGF- $\beta$ 1 with blocking antibody experiments. Since TGF- $\beta$ 1 mRNA was detected in all 3 RMS cell lines and increased after treatment with TGF- $\beta$ 1 in 2 of them, suggesting an autocrine role for TGF- $\beta$ 1, lack of detection of endogenous TGF- $\beta$ 1 function may be due to an inactive form of endogenous TGF- $\beta$ 1, which is usually the case in tumor models <i>in vitro</i> . To clarify this issue, TGF- $\beta$ 1 protein levels will be evaluated in conditioned media from all 3 cell lines with the mink lung fibroblast (CCL-64) bioassay. The effects of TGF- $\beta$ 1 on the cell proliferation will be analyzed in conjunction with changes concerning mRNA levels of cell cycle related genes, such as c-myc, c-fos and c-jun. Differentiation will be evaluated by morphology (cell fusion assay), as well as detection of possible changes in the mRNA levels of the muscle determination genes, MyoD1 and Myogenin and the muscle protein genes desmin and CK-MM. Muscle protein expression will be evaluated by immunofluorescence using commercially available antibodies.		

Publications:

McCune BK, Patterson K, Chandra RS, Kapur S, Sporn M, Tsokos M. Expression of transforming growth factor- $\beta$  isoforms in small round cell tumors of childhood: An immunohistochemical study. *Am J Pathol* 1993;142:49-58.

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

PROJECT NUMBER

Z01 CB 09370-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Detection of p53 Mutations in Solid 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	LP NCI
OTHER:	M. Quezado	Fogarty Fellow	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1

PROFESSIONAL:

3/4

OTHER:

1/4

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.)

p53 mutations have been detected in many tumors and interpreted as a late event in tumorigenesis, probably involved in tumor progression rather than tumor development. Recent studies have demonstrated the presence of germline p53 mutations in some Li-Fraumeni pedigrees and hence a role of p53 mutations in hereditary susceptibility to human cancer. Rhabdomyosarcoma, a common soft tissue sarcoma in the families with the Li-Fraumeni syndrome, was found to have p53 mutations. However, the frequency of such mutations in pediatric tumors remains unknown. We have recently established a non-radioisotopic method to detect mutations. This method is based on the formation of DNA hetero-duplexes with variable motility in mutation detection enhancement (MDE) gels. Mutations can thus be detected in PCR-amplified DNA extracted even from paraffin embedded tissues. Using this method, we have studied PCR fragments spanning exons 4, 5, 6, 7 and 8 of the p53 gene from 13 rhabdomyosarcomas (RMS) (mostly paraffin blocks). A high percentage of the studied tumors (46%) exhibited p53 mutations, more often in exon 7, followed by exons 8 and 5. The presence of mutations was confirmed in some cases with the standard single stranded conformational polymorphism (SSCP) method and with a direct sequencing method of single stranded DNA obtained by asymmetric PCR. We have currently expanded the study to include a larger number of RMS, as well as other solid pediatric tumors, such as neuroblastoma and peripheral and central PNET/Ewing's sarcoma cases. The survival of patients with mutated and non-mutated tumors will be compared to explore a potential prognostic role of p53 mutations in this group of tumors.

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

PROJECT NUMBER

201 CB 09379-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Reverse Transcriptase (RT)-PCR Distinguishing Pediatric Tumors with t(11;22)

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

PI:	M. Tsokos	Chief, Ultrastructural Pathology Section	LP NCI
OTHER:	M. Quezado	Fogarty Fellow	LP NCI
	M. Abaza	Guest Researcher	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1

PROFESSIONAL:

1

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.)

The distinction of peripheral primitive neuroectodermal tumors (PNET) from other tumors of neuroectodermal origin, such as neuroblastoma and central PNET, or even rhabdomyosarcoma is not always easy. Although a specific cytogenetic abnormality, t(11;22) (q24;q12), characterizes peripheral PNET, it is not always feasible to perform cytogenetic analysis for diagnostic purposes. The recent identification of transcribed sequences from the breakpoint regions and the detection of abnormal hybrid transcripts in those cases with the translocation has led to the use of RT-PCR as a sensitive diagnostic method to demonstrate specific fusion transcripts in those tumors with t(11;22). We have employed the RT-PCR technique to detect abnormal transcripts in a number of pediatric solid tumors and cell lines. Our preliminary data have shown specific expression of abnormal transcripts only by the peripheral PNET and not central PNET or neuroblastomas. The specificity of the method will be tested on a larger number of tumors.

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

PROJECT NUMBER

701 CB 09384-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Diagnostic Electron Microscopy and Clinically Applied Research

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

PI:	M. Tsokos	Chief, Ultrastructural Pathology Section	LP NCI
OTHER:	Y. Hijazi	Expert	LP NCI
	J. Jefferson	Histopath. Technician	LP NCI
	C. Brown	Biol. Lab. Technician	LP NCI
	S. Mims	Biologist	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

4

PROFESSIONAL:

3

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

A

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

The Ultrastructural Pathology (UP) Section provides specialized diagnostic services for cases difficult to classify with conventional methods. In addition, the UP section has a long-term commitment to: improve available diagnostic techniques for the classification of poorly differentiated solid pediatric tumors and to define prognostic factors which may lead to development of new therapeutic strategies. For this reason, the section has undertaken independent research projects (reported separately) regarding: 1) the role of growth factors in the differentiation and proliferation of pediatric tumors *in vitro*; 2) the identification of subgroups with p53 mutations or high mdm gene expression which may have prognostic significance and 3) the use of non-conventional diagnostic methods, such as the reverse polymerase chain reaction (RT-PCR) for more accurate characterization of certain tumors.

The UP section also provides diagnostic consultative services to the Pediatric Oncology Branch (POB) at the NCI and has collaborative projects with both the POB and other investigators in the Clinical Center.

Examples of clinicopathologic projects directed by the UP Section are 1) P-glycoprotein expression in Ewing's sarcoma and peripheral primitive neuroectodermal tumor (PNET) before and after treatment; 2) Proliferating cell nuclear antigen as a prognostic factor in childhood rhabdomyosarcoma; 3) Ewing's sarcoma versus PNET: does histology play a prognostic role?

Examples of projects in which the UP Section has a collaborative role are: 1) differentiation of rhabdomyosarcoma cell lines with Ara-C; 2) expression of insulin-growth factor (IGF) II in rhabdomyosarcoma cells by *in situ* hybridization; 3) tumor regression after treatment with a monoclonal anti-IGF II antibody in a rhabdomyosarcoma nude mouse model; 4) possible induction of differentiation and/or increased levels of  $\beta_2$ -microglobulin after treatment of refractory neuroblastoma with tumor infiltrating lymphocytes, interferon gamma and interleukin-2; 5) evaluation of the role of cytoskeletal proteins (tubulin and vimentin) in the development of cisplatin resistance and 6) mechanisms of taxol-induced cell death in Chinese hamster V79 cells.

Major Findings:

In 1992, 160 cases were submitted for diagnostic electron microscopic examination. The majority of the cases (over 80%) were completed (sectioned and photographed) and reports were generated as "Supplemental Electron Microscopy Reports". The electron microscopy (EM) reports are generated through the MIS detection system and copies are kept in the Surgical Pathology files (attached to the surgical pathology reports), and in the EM files (in the patient's folders and in a notebook). In very few cases, the ultrastructural findings are included in the surgical pathology report, in which reference to the corresponding EM number is made. The few cases that are not reported are cases submitted for block only (pending review of the H&E slides), study cases, or teaching cases (submitted only out of personal interest). All the data from the diagnostic cases (patient's name, number, status of the case, and diagnosis) are recorded in a log book in the UP Section, that serves as the working book, and in the data base files in a computer.

The turnaround time for completion of each case varies according to the clinical needs. Some cases are processed and photographed in 24 hours (rush cases) and a report follows soon after, others in 4 days (priority, but not rush cases), and others in 7-10 days (routine cases). The status of a case can be modified (e.g. from routine to priority, or from block only to routine) at any time during the processing period, according to the physician's and pathologist's needs.

The UP Section also owns a frozen tissue bank and various tumor cell lines from pediatric tumors. The frozen tissues serve as material for diagnostic (immunocytochemistry with certain monoclonal antibodies) or research (RNA, DNA extraction) studies. The established cell lines are submitted for cytogenetic analysis that can occasionally serve for final diagnosis (e.g. the characteristic 11/22 translocation in Ewing's sarcoma and primitive neuroectodermal tumor), and comprise the material for the research projects of the section.

Publications:

Tsokos M. Peripheral primitive neuroectodermal tumors - diagnosis, classification and prognosis. In: Rosenberg H, Bernstein J, eds. Perspectives in pediatric pathology. Basel, Karger, 1992;16:27-98.

Crouch GD, Kalebic T, Tsokos M, Helman LJ. Ara-C treatment leads to differentiation and reverses the transformed phenotype in a human rhabdomyosarcoma cell line. Exp Cell Res 1993;204:210-16.

Hijazi YM, Axiotis CA, Navarro S, Steinberg SM, Horowitz ME, Tsokos M. Immunohistochemical detection of P-glycoprotein in Ewing's sarcoma and peripheral primitive neuroectodermal tumors before and after chemotherapy. Am J Clin Pathol (in press)

Minniti CP, Tsokos M, Newton WA Jr, Helman LJ. Specific expression of insulin-like growth factor-II in rhabdomyosarcoma tumor cells. Am J Clin Pathol (in press)

Horowitz ME, Miser JS, Wexler LH, Belasco J, Triche TJ, Tsokos M, Steinberg SM, McClure L, Glatstein E, Pizzo PA, Kinsella T. Total body irradiation and autologous bone marrow transplant in the treatment of high risk Ewing's sarcoma and rhabdomyosarcoma. J Clin Oncol (in press)

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

PROJECT NUMBER

Z01 CB 09172-05 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Cellular Interactions with Thrombospondin

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

PI:	D. Roberts	Chief, Biochemical Pathology Section	LP NCI
OTHER:	V. Zabrenetzky	Staff Fellow	LP NCI
	N. Guo	Visiting Associate	LP NCI
	E. Negre	Visiting Fellow	LP NCI

COOPERATING UNITS (if any)

Howard Fillit, Mount Sinai Medical Center; Patricia Steeg, Lab. of Path., NCI;  
Rachael Yabkovicz, Univ. of Michigan; Diane Blake, Meharry Medical College

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

2.9

OTHER:

0.6

CHECK APPROPRIATE BOXES)

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

B

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

The functions of thrombospondin in cell adhesion and migration and tumor metastasis 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 that 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 (Cancer Res 48:1988:6875). An unusual sulfated glycolipid present only in melanoma cell lines that spread on thrombospondin binds to thrombospondin and mediates melanoma cell spreading (ibid.). To further define the mechanism of thrombospondin interactions with tumor cells, receptors for the carboxyl-terminus of thrombospondin are being characterized. Synthetic peptides from this region of thrombospondin are being used to define the sites recognized by thrombospondin receptors. The intracellular responses of cells to binding of thrombospondin to the two types of receptors are also being investigated. Expression of thrombospondin mRNA in tumor cells is being examined to look for association of thrombospondin synthesis with *in vivo* metastatic potential.



Major Findings:

We have prepared peptides from the three type I repeats of human endothelial cell thrombospondin, containing the consensus sequence - Trp-Ser-Xaa-Trp-, that bind to sulfated glycoconjugates including heparin and sulfatide. The peptides are potent inhibitors for the binding of thrombospondin, laminin, or apolipoprotein E to these ligands. The thrombospondin peptides promote melanoma cell adhesion when immobilized on plastic and inhibit heparin-dependent binding of thrombospondin or laminin to human melanoma cells. The active peptides lack any previously identified heparin-binding consensus sequences and most do not contain any basic amino acids. The two Trp residues and the Ser residue are essential. The Trp residues must be spaced less than four residues apart. The Pro residue is essential for activity of the pentapeptide Trp-Ser-Pro-Trp-Ser, but some larger peptides with substitutions of the Pro residue are active. Peptides containing the consensus sequence and basic amino acids are chemotactic for A2058 human melanoma cells. The functional importance of this novel heparin and sulfatide binding motif is suggested by its conservation in other members of the thrombospondin gene family, complement components, and in many members of the cytokine receptor and transforming growth factor  $\beta$  superfamilies. The type I peptides of thrombospondin thus define a new class of heparin-binding peptides.

Recently, we found that a related family of peptides from the type I repeats of thrombospondin bind avidly to fibronectin. These peptides bind specifically to the collagen binding domain of fibronectin and inhibit binding of fibronectin to gelatin or native type I collagen. The peptides specifically inhibit fibronectin mediated tumor cell adhesion to collagen but not direct adhesion to fibronectin. These peptides are therefore a novel agent to disrupt fibronectin mediated cell adhesion to matrix collagen.

We have shown that tumor cell thrombospondin steady state mRNA levels and protein secretion are inversely related to malignant progression. In the K-1735 murine melanoma, the low metastatic Clone 19 cell line contained high levels of TSP mRNA levels and the high metastatic TK cell line had none. The highly metastatic MDA-MB-435 human breast cell line had only one third the level of TSP mRNA expressed in the non-metastatic and estrogen-dependent MCF-7 cell line. An immortalized human bronchial epithelial cell line (BEAS-2B) contained higher levels of TSP mRNA levels compared to three ras transformants. Cell lines derived from subcutaneous tumors of each ras transformant, which exhibited increased tumorigenic and metastatic behavior *in vivo*, contained further reductions in TSP mRNA levels. Expression of the extracellular matrix molecule fibronectin was not correlated with malignant progression in these cell lines. Thus, in a variety of tumor cell types, malignant progression is specifically associated with reduced expression of TSP mRNA.

Publications:

Guo N, Krutzsch HC, Negre E, Vogel T, Blake DA, Roberts, DD. Heparin- and sulfatide-binding peptides from the type I repeats of thrombospondin promote melanoma cell adhesion. Proc Natl Acad Sci USA 1992;89:3040-4.

Buée L, Roberts DD, Hof PR, Delacourte A, Morrison JH, Fillit HM. Immunohistochemical identification of thrombospondin in normal brain and the amyloid deposits of Alzheimer's disease. *Am J Pathol* 1992;141:783-8.

Guo N, Krutzsch HC, Nègre E, Zabrenetzky VS, Roberts DD. Heparin-binding peptides from the type I repeats of thrombospondin: structural requirements for heparin binding and promotion of melanoma cell adhesion and chemotaxis. *J Biol Chem* 1992;267:19349-55.

Roberts DD. Interactions of thrombospondin with sulfatides and other sulfated glycoconjugates. In: Lahav J, ed. *Thrombospondin*. CRC Press (in press)

Sipes JM, Guo N, Nègre E, Vogel T, Krutzsch HC, Roberts DD. Inhibition of fibronectin binding and fibronectin-mediated cell adhesion to collagen by a peptide from the second type I repeat of thrombospondin. *J Cell Biol* (in press)

Yabkowitz R, Guo N, Roberts DD, Shimizu Y. T cell adhesion to thrombospondin mediated in part by VLA-4 and VLA-5 integrins. *J Immunol* (in press)

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

PROJECT NUMBER

Z01 CB 09173-05 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Roberts Chief, Biochemical Pathology Section LP NCI  
 OTHER: E. Negre Visiting Fellow LP NCI

COOPERATING UNITS (if any)

T. Walsh, Pediatric Oncology, NCI, NIH

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF 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 unreduced type. Do not exceed the space provided.)

Adhesive specificities of *Staphylococcus aureus*, some *Enterococcus* species, *Candida albicans*, and elementary bodies of *Chlamydia trachomatis* are being examined. These have been screened for binding to extracellular matrix proteins and glycoproteins and glycolipids of known structure and to glycoconjugates isolated from target tissues to which the pathogens adhere. Where possible, inhibitors of each binding specificity will be identified using the solid phase assays and then tested using *in vitro* cytoadherence assays and *in vivo* infection assays to determine the role of each in cytoadherence and initiation of infection.

Major Findings:

We are studying the binding of fibronectin and recombinant and proteolytic fragments of this protein to *Candida albicans* to determine which of the numerous functional domains of the protein are involved in the interactions between the yeast and the protein. *In vitro* assays were developed to quantify the attachment of *C. albicans* to fibronectin or the fragments coated on an insoluble synthetic matrix, and to evaluate the binding of soluble fibronectin and the fragments to *C. albicans* in suspension. *C. albicans* culture has been the critical step to obtain reproductive results. Expression of the receptor depends on growth medium, temperature, shear, and time in culture. Optimal conditions have been established to obtain high receptor expression. Binding of labeled fibronectin to *C. albicans* is saturable and reversible but requires an unusual 3 hours to reach equilibrium. Equilibrium binding analysis indicated 2 classes of sites with  $K_d = 1.3 \times 10^{-9}$  M,  $n = 5000$  sites/cell and  $K_d = 1.2 \times 10^{-7}$  M,  $n = 30100$  sites/cell, respectively. In contrast to the published literature, binding is not inhibited by Arg-Gly-Asp peptides but requires novel sites on interaction between *C. albicans* and the heparin- and collagen-binding domains of fibronectin. In contrast to integrin-mediated binding of mammalian cells to fibronectin, binding to *C. albicans* does not require divalent cations and is enhanced in the presence of EDTA. Therefore, binding of fibronectin to *C. albicans* is mediated by several sites on the protein and probably does not involve the classical integrin-fibronectin interactions.

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

PROJECT NUMBER

201 CB 09174-05 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Roberts	Chief, Biochemical Pathology Section	LP NCI
OTHER:	N. Guo	Visiting Fellow	LP NCI
	H. Yu	Visiting Associate	LP NCI

COOPERATING UNITS (if any)

H. Gralnick, Hematology Service, CC, NIH; J. Cashel, Biochemical Pathology Section, Lab. of Path., NCI; H. Fillit, Mount Sinai Medical Center

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.0

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.)

We have found that sulfated glycolipids participate in tumor cell adhesion by directly promoting adhesion (Cancer Res 1988;48:3367) and as receptors for thrombospondin on melanoma cells (Cancer Res 1988;48:6785). 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. The molecular basis of binding of adhesive glycoproteins to sulfatide and heparan sulfate proteoglycans are being investigated by identifying sequences in these molecules responsible for binding. Both proteolytic and recombinant fragments of the adhesive proteins are being used to map the active binding domains.

Major Findings:

We have characterized the interaction of thrombospondin and laminin with purified heparan sulfate proteoglycan. Murine EHS tumor laminin and human platelet thrombospondin bound with high affinity to a vascular heparan sulfate proteoglycan purified from bovine kidney. Binding of thrombospondin was comparable to the isolated heparan sulfate chains and the intact proteoglycan and did not require the core protein. Thrombospondin binding to the intact proteoglycan, heparan sulfate, or heparin-bovine serum albumin immobilized on plastic was heterogeneous with at least two classes of binding affinities. Recombinant heparin-binding domain from thrombospondin also bound specifically to the proteoglycan, heparin BSA, and sulfatide. Binding of laminin to the HSPG or heparin at 2°C could be described by a single class of high affinity binding sites. At higher temperatures, however, the Scatchard plots were concave downward. Temperature-dependent apparent cooperative binding was also observed for laminin binding to sulfatide. The binding data could not be fit by a positive cooperative binding model, but probably results from ligand induced oligomerization of the laminin at higher temperatures. Approximately 60% of thrombospondin or laminin binding to cultured bovine aortic endothelial cells was heparin dependent. The cells expressed proteoglycans that bound thrombospondin and laminin and cross reacted with a monoclonal antibody to the core protein of the kidney proteoglycan.

A laminin-binding peptide (peptide G), predicted from the cDNA sequence for a 33 kDa protein related to the 67 kDa laminin receptor, specifically inhibits binding of laminin to heparin and sulfatide. Since the peptide binds directly to heparin and inhibits interaction of another heparin-binding protein with the same sulfated ligands, this inhibition is due to direct competition for binding to sulfated glycoconjugates rather than an indirect effect of interaction with the binding site on laminin for the 67 kDa receptor. Direct binding of laminin to the peptide is also inhibited by heparin. This interaction may result from contamination of the laminin with heparan sulfate, as binding is enhanced by addition of substoichiometric amounts of heparin but inhibited by excess heparin and two heparin-binding proteins. Adhesion of A2058 melanoma cells on immobilized peptide G is also heparin-dependent, whereas adhesion of the cells on laminin is not. Antibodies to the  $\beta 1$  integrin chain or laminin block adhesion of the melanoma cells to laminin but not to peptide G. Thus, the reported inhibition of melanoma cell adhesion to endothelial cells by peptide G may result from inhibition of binding of laminin or other proteins to sulfated glycoconjugate receptors rather than from specific inhibition of laminin binding to the 67 kDa receptor.

Publications:

Guo N, Kruttsch HC, Vogel T, Roberts DD. Interactions of a laminin-binding peptide from the 67-kDa laminin receptor with laminin and melanoma cells are heparin-dependent. *J Biol Chem*;267:17743-7.

Roberts DD, Mecham RP, eds. Cell surface glycoconjugates: structure and function. Academic Press (in press)

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

PROJECT NUMBER

Z01 CB 09175-05 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Glycoconjugate Antigens Expressed in Cancer Cells

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

PI: D. Roberts Chief, Biochemical Pathology Section LP NCI  
 OTHER: H. Yu Visiting Associate LP NCI

COOPERATING UNITS (if any)

R. Goldblum, Department of Pediatrics, University of Texas Medical Branch,  
 Galveston; S. Yedgar, Hebrew University, Jerusalem

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF 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 unredacted type. Do not exceed the space provided.)

Altered glycosylation of glycolipids and glycoproteins is frequently observed in tumor tissues and cultured cancer cell lines. Changes in expression of glycosyltransferases produce unique carbohydrate structures which can be used to differentiate tumor from normal tissue and in some cases alter the biological activity of adhesive proteins and receptors which bear the altered oligosaccharides. We are examining the specificity of some monoclonal antibodies that recognize oligosaccharide determinants on functionally important molecules and the structures of sulfated glycoconjugates produced by cancer cells which bind to adhesive proteins such as laminin and thrombospondin. Currently we are examining globo-series sulfated glycolipids produced in human breast cancer and carbohydrates recognized by monoclonal antibodies to human secretory component and sialyl Lewis a which recognize glycoproteins and glycolipids produced in colon adenocarcinomas.

Major Findings:

The monoclonal antibody 19-9 recognizes carbohydrate antigens on glycolipids and mucins produced by adenocarcinomas. SW-1116 colon adenocarcinoma cells constitutively secrete mucin containing this epitope. Secretion is independent of cAMP level but can be stimulated by the Ca ionophore A23187. Arachidonic acid and its metabolites inhibit secretion. Electron microscopic studies reveal mucin near the plasma membrane and in vesicular structures. The control of secretion of mucin by SW1116 cells is relevant to pathological states associated with excessive constitutive secretion of mucin including gastrointestinal cancer and cystic fibrosis.

Publications:

Yedgar S, Eidelman O, Molden E, Roberts DD, Etcheberrigaray R, Goping G, Pollard HB. cAMP-independent secretion of mucin by SW1116 human colon carcinoma cells: differential control by calcium ionophore A23187 and arachidonic acid. *Biochem J* 1992;283:421-6.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 CB 00891-10 LP
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Stimulated Motility in Tumor Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	M. Stracke Sr. Staff Fellow	LP NCI
	L. Liotta Chief, Tumor Invasion and Metastases Section	LP NCI
OTHER:	E. Schiffmann Scientist Emeritus	LP NCI
	Å. Arestad Special Volunteer	LP NCI
	J. Murata Visiting Fellow	LP NCI
	H. Krutzsch Research Chemist	LP NCI
COOPERATING UNITS (if any) R. Hopkins and P. Harley, PRI/Dyne Corp., FCRDC; M. Sveta, OTC/Biotechnology Research Division, Gaithersburg, MD		
LAB/BRANCH Laboratory of Pathology		
SECTION Tumor Invasion and Metastases Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL STAFF YEARS: 4.5	PROFESSIONAL: 3.0	OTHER: 1.5
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		
B		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)		
<p>We have been studying tumor cell motility as a component of the process of metastatic dissemination. A number of autocrine motility factors (AMF's) have been shown to be synthesized by human tumor cells. These AMF's stimulate both directed and random motility in the same cells that produce the factor. Recently, we have purified a new AMF to homogeneity and have named this factor autotaxin (ATX). ATX is a basic glycoprotein with a molecular weight of 120,000 daltons and a pI ~ 7.8. It stimulates pertussis-toxin sensitive motility in tumor cells when present at concentrations in the picomolar to nanomolar range. Anti-peptide antibodies, which recognize the protein in immunoblots, have been produced in rabbits. Oligonucleotides have been synthesized which correspond to the peptide sequences and have been used both as primers in polymerase chain reaction (PCR) amplifications and as probes to screen cDNA libraries made from the same melanoma cell line. In addition, we are continuing to characterize the protein and its active site by studies with endoglycosidases to identify the nature of the linked sugar moieties.</p>		

## Major Findings:

### I. Anti-Peptide Antibodies

The 120,000 dalton ATX has been purified to homogeneity. Amino acid sequence has been obtained on 19 peptides, comprised of 215 amino acids. Several of these peptide sequences have been synthesized, cross-linked to bovine serum albumin, and used to immunize rabbits. Anti-serum was purified by ammonium sulfate precipitation followed by peptide affinity chromatography. Three of these purified antibodies, made against two different ATX peptides, were found to bind to a single 120 kDa protein on immunoblots. A fourth anti-peptide antibody, made against a third peptide, has proven difficult to maintain over a long period of time in rabbits. To date, none of the antibodies either immunoprecipitates or neutralizes activity. However, one of the anti-peptide antibodies has been used for immunohistochemical staining of the melanoma cells (A2058). These stains reveal predominant perinuclear and cytosolic localization, consistent with a secreted protein. Similar preliminary staining is now being done with human tumor sections.

### II. Cloning of Autotaxin (ATX)

The primary goal of the motility group is to clone the gene for ATX. Because ATX is made by A2058 cells in small quantities and because the mRNA for ATX appears to be rare on northern blots, we are approaching this goal by using a number of complementary approaches. Attempts to utilize oligonucleotides derived from the peptide amino acid sequence have proven problematic because of the inherently high level of codon degeneracy. We are now utilizing an affinity-purified anti-peptide antibody to screen an A2058 cDNA library for the ATX gene. We have isolated six clones whose fusion proteins react with the ATX anti-peptide antibody. These cDNA inserts have been amplified using PCR techniques, digested with appropriate restriction enzymes, and ligated into plasmids for DNA sequence analysis. In addition to sequencing, these plasmids will be used as templates for PCR analysis with various projected ATX oligonucleotides as probes. Northern blot analysis of poly-T-purified A2058 RNA, using labelled inserts as probes, will be performed to see whether the clones react with an appropriate size mRNA. Further studies with the labelled inserts as probes include southern blot analysis to see whether the different clones are cross-reactive and, therefore, related.

### III. Studies of ATX Glycosylation

During the course of purifying ATX, several lines of evidence indicated that the protein was glycosylated. Its fuzzy appearance when subjected to SDS polyacrylamide gel electrophoresis suggested variable glycosylation; this was further indicated on two dimensional gels which were consistent with multiple neutral glycosylation states. The protein bound to concanavalin A lectin affinity columns and was eluted off by an appropriate mannose sugar solution. In addition, sequence information was consistent with the presence of sugar residues on certain amino acids.

We have now further characterized ATX as a glycoprotein. Several lectins bind to immobilized ATX including concanavalin A (mannose), SNA [ $\alpha$ (2-6)-linked terminal sialic acid], and MAA [ $\alpha$ (2-3)-linked terminal sialic acid]. When ATX is treated with neuraminidase to remove terminal sialic acid moieties, the molecular weight of ATX decreases by 1000-2000 Da. Neither SNA or MAA any longer bind to ATX, but Con A still does. When ATX is treated with N-Glycosidase F, which removes N-linked sugars at the asparagine residue, the molecular weight of ATX decreases to approximately 100 kDa, a loss of 20 kDa or approximately 16% of the total molecular weight. None of the lectins any longer binds to this treated ATX. Furthermore, when ATX is treated with trifluoromethane sulfonic acid which removes 80-90% of all sugar moieties, the molecular weight of ATX decreases to approximately 105 kDa.

These data are consistent with a predominantly N-linked glycosylation of ATX with any O-linkage accounting for less than 10% of total sugar moieties. It is not yet clear whether this glycosylation plays a role in cellular activation by ATX or whether it serves primarily in the cellular regulation of ATX secretion.

#### Plans for Future Studies:

Our primary goal remains to clone the autotaxin gene. We plan to utilize multiple techniques to achieve this goal, including both antibody and oligonucleotide screening of cDNA libraries. We are continuing to purify ATX from A2058 conditioned medium and hope to get additional peptide sequences in the near future. We plan to digest the purified ATX with rare-cutting proteases so that the novel peptide sequences will be as long as possible. These longer peptide sequences would allow us to synthesize longer oligonucleotides and run the library screens under more stringent conditions. We also plan to study various cellular activators, such as phorbol esters and TGF $\beta$ , to see if these will increase ATX synthesis in A2058 cells. Agents that increase the synthesis of ATX will be presumed to increase mRNA production. A cDNA library made utilizing these agents will therefore have ATX as a more frequent transcript.

Once the A2058 gene is cloned, we would like to put it into various expression vectors and see if we can produce motility-stimulating activity. The large scale production of protein would allow us to perform ATX active site analysis and to study the ATX cell surface receptor. Knowing the gene sequence would allow us to perform northern and southern blot analyses of cells collected under varying conditions, look at gene regulation, and perform *in situ* hybridizations of tumor tissue.

We plan to perform preliminary studies of the ATX receptor using purified protein. ATX can be cross-linked to the receptor and analyzed by immunoblot, using anti-peptide antibodies. Binding affinity can be estimated by radiolabelling ATX and performing Scatchard plot analysis. Radiolabelled ATX can also be cross-linked to the cell surface receptor to be separated by polyacrylamide gel electrophoresis. These relatively simple experiments would allow us to estimate the size of the receptor, to determine whether it is composed of a single peptide or multiple subunits, and to approximate the number of receptors present per cell.

Purified ATX will also be used to continue the glycosylation studies. Knowing that the sugar moieties are predominantly N-linked, we can begin to analyze the composition of the glycan groups. We will also attempt to assess what role these sugar groups play in ATX-stimulated motility.

Finally, the ATX anti-peptide antibodies will be used to perform immunohistochemical studies on human tumor tissues and on murine embryonic tissue. These studies will evaluate possible roles for this motility factor in metastasis and in development, respectively. If the antibodies proved to be tumor specific, they could be used in potential treatment modalities, such as ricin-tagged antibodies to eliminate potentially metastatic cells.

#### Publications:

Stracke ML, Krutzsch HC, Unsworth EJ, Årestad A, Cioce V, Schiffmann E, Liotta LA. Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. *J Biol Chem* 1992;267:2524-9.

Stracke ML, Liotta LA. Multi-step cascade of tumor cell metastasis. *In Vivo* 1992;6:309-16.

Margulies IMK, Höyhty M, Evans C, Stracke ML, Liotta LA, Stetler-Stevenson WG. Urinary type IV collagenase: elevated levels are associated with bladder transitional cell carcinoma. *Cancer Epidemiol, Biomarkers & Prevention* 1992;1:467-74.

Stracke ML, Soroush M, Liotta LA, Schiffmann E. Cytoskeletal agents inhibit motility and adherence of human tumor cells. *Kidney Int* 1993;43:151-7.

Stracke ML, Liotta LA, Schiffmann E. The role of autotaxin and other motility-stimulating factors in the regulation of tumor cell motility. (in press)

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

PROJECT NUMBER

Z01 CB 00892-10 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. This 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. Steeg	Research Biologist	LP NCI
OTHER:	N. MacDonald	Visiting Fellow	LP NCI
	U. Flatow	Biologist	LP NCI
	A. De La Rosa	Visiting Fellow	LP NCI
	A. Warren	Guest Researcher	LP NCI

COOPERATING UNITS (if any)

Molecular Oncology, Inc., Gaithersburg, MD (CRADA); Dr. H. Westphal, NEI; Dr. W. Bohn, NIA; Dr. B. Zetter, Harvard Med. Sch.; Dr. M. Bissel, U. Cal., Berkeley

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.6

PROFESSIONAL:

2.7

OTHER:

0.9

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 role of *nm23* in the regulation of tumor metastasis has been investigated. Transfection of human *nm23-H1* cDNA into the human MDA-MB-435 breast carcinoma cell line reduced the *in vivo* metastatic potential of this cell line by 50-90%. The *nm23-H1* transfectants exhibited less colonization potential in soft agar, and reduced motility responses to serum, IGF or PDGF *in vitro*. The data establish the metastasis suppressive activity of the human *nm23-H1* gene in a human breast tumor cell line.

Two studies have indicated that Nm23 is involved in the normal differentiation process. Immunohistochemical evaluation of NM23 expression indicated its increased expression coincident with the functional differentiation of the murine heart, nervous system and most epithelial tissues in embryogenesis. *In vitro* analysis of control and *nm23-H1* transfected MDA-MB-435 cell lines indicates that *nm23-H1* can directly induce certain aspects of the structural and functional differentiation of normal mammary ducts.

A novel biochemical function has been identified for NM23 protein based on a novel serine phosphorylation as a cAMP regulated ATPase. Examination of control and *nm23-1* transfected murine melanoma cell lines indicates that the serine phosphorylation of NM23 is directly correlated with *nm23-1* suppression of metastasis, while the previously identified nucleoside diphosphate kinase activity of NM23 was not.

NM23 transfectants in murine melanoma, human breast carcinoma and human ovarian carcinoma cell lines were more sensitive to the growth inhibitory effects of cisplatin than were control transfectants. Cisplatin inhibited metastasis formation of *nm23-1* transfected murine melanoma cells *in vivo* to a greater extent than control transfectants. The data indicate the potential use of NM23 expression to reduced metastatic colonization and improve chemotherapeutic efficacy.

Major Findings:

The *nm23* family of genes was discovered in my laboratory on the basis of its reduced expression in highly metastatic murine melanoma cell lines, as compared to related but low metastatic potential melanoma cell lines. To date, histopathologic or clinical course correlates of aggressive disease have been significantly correlated with reduced *nm23* expression in cohorts of human breast, hepatocellular and gastric carcinomas and melanoma, and with *nm23* mutations/structural alterations in colorectal carcinoma and neuroblastoma. Transfection of murine *nm23-1* cDNA into the murine K-1735 TK melanoma cell line inhibited *in vivo* metastatic potential by 50-90%.

Transfection of a human *nm23* cDNA into a human breast carcinoma cell line has been completed. The *nm23-H1* cDNA linked to a constitutive CMV promoter, was transfected into the human MDA-MB-435 breast carcinoma cell line. Both bulk transfected cultures and stable, high expression clonal lines were tested against control lines transfected with an empty CMV construct. An *in vivo* metastasis assay was developed that is highly comparable to the clinical course of the human disease: A relatively small number of breast carcinoma cells are injected into the mammary fat pad (mpf) of nude mice, a primary tumor develops in the mammary gland, where tumor cell-stromal interactions and locally produced growth factors can be operative, and metastases form in the draining lymph nodes and lungs of approximately 50% of animals injected. The *nm23-H1* transfectants formed primary mammary tumors that were of similar size to the control transfectants, but produced metastases in 50-90% fewer animals than the control transfectants. The inhibition of *in vivo* metastatic potential was associated with altered function in several *in vitro* assays: (1) *nm23-H1* transfectants exhibited a reduced colonization in soft agar as compared to control transfectants. This assay may be most indicative of the ability of tumor cells to colonize at a distant site, apart from mammary gland derived growth factors and stromal cell interactions. (2) Recent research indicates that the most successful metastatic tumor cells may be those that are stimulated to colonize by widely available cytokines. In this regard, control transfectants were 2-4-fold stimulated by TGF- $\beta$  in colonization assays, while the *nm23-H1* transfectants were not. (3) The *in vitro* motility of the control and *nm23-H1* transfectants was determined in blinded experiments by Dr. Bruce Zetter, Harvard Medical School. Control transfectants migrated in response to serum, PDGF and IGF. The *nm23-H1* transfectants failed to migrate to any of these attractants. The unstimulated motility of the *nm23-H1* and control transfectants were equivalent, indicating that *nm23-H1* acts to inhibit the signal transduction process. Additional transfection experiments are underway using a human ovarian carcinoma cell line.

I have visited two pharmaceutical firms to discuss using the MDA-MB-435 cell line in a drug screening operation to identify agents that stimulate tumor cells to re-express Nm23. Toward this goal, the promoter regions of the human *nm23-H1* and *nm23-H2* genes are being identified, for potential use in a luciferase reporter assay. Also, antibodies to Nm23 proteins are being further developed and characterized. Interviews with additional companies are planned before CRADA and licensing negotiations commence.

The normal function of Nm23 has been speculated to involved development and differentiation, based on research into its *Drosophila* homologue, *awd*. Mutation or reduced expression of *awd* resulted in aberrant differentiation of multiple tissues when presumptive adult tissues in the fly imaginal discs attempted to divide and differentiate post-metamorphosis. In collaboration with Dr. Heiner Westphal, we have developed the first evidence for an association of Nm23 expression and development and differentiation in a mammalian system. Immunoperoxidase staining of mouse embryos at every day of embryonic development was conducted. Nm23 expression was uniformly low until the onset of organogenesis. Increased Nm23 expression was correlated with the functional differentiation of the heart, nervous system and virtually every epithelial tissue in embryogenesis, and the mammary gland in nulliparous adults. A study underway in the laboratory of Dr. Mina Bissel, UC Berkeley, has confirmed and extended these findings. Dr. Bissel has pioneered *in vitro* assays using extracellular matrices as growth supports, which enable normal mammary epithelial cells to structurally and functionally differentiate into ducts. Her lab examined the control- and *nm23-H1* transfected MDA-MB-435 transfectants in a blinded manner, and has found that *nm23-H1* expression induced several aspects of the normal phenotype in this malignant tumor cell line, including ductal morphology, production and secretion of a basement membrane and production of sialomucins.

In order to understand the suppressive effects of *nm23* on tumor metastatic potential, the biochemical mechanism of its action must be identified. Furthermore, in a drug screening assay, it is imperative to know that an agent both stimulates Nm23 expression as well as its functional activity. The Nm23 proteins have been known to possess nucleoside diphosphate kinase (NDPK) activity, in which a terminal phosphate is removed from ATP, transferred to a histidine residue on Nm23, and ultimately transferred to a NDP to recreate an NTP. Several observations from my laboratory and other laboratories have cast doubt as to whether the NDPK activity of Nm23 is responsible for its biological suppressive effects.

My laboratory has identified a new biochemical pathway for Nm23 as a cAMP regulated ATPase. This pathway is based on the identification of a novel phosphorylation of Nm23. Immunoprecipitated Nm23 as well as purified recombinant Nm23 autophosphorylated *in vitro* in an acid stable, base labile manner, which is inconsistent with a phosphohistidine. Phosphoamino acid analysis indicated a phosphoserine on Nm23. This is a low energy phosphorylation, consistent with the acid/base stability data, and is thermodynamically incapable of participating in the phosphate transfer of the NDPK reaction. An acid stable (serine) Nm23 phosphorylation was also observed *in vivo* in human breast carcinoma and murine melanoma cells by [<sup>32</sup>P]orthophosphate labeling. Formation of the acid stable (serine) phosphorylated Nm23 was inhibited by cAMP *in vitro* and forskolin *in vivo*, suggesting that this pathway is regulated in the signal transduction process. No effect of cAMP was observed on the Nm23-NDPK activity. Once phosphorylated, Nm23-phosphoserine can release free phosphate in response to NTPs, NDPs and inactive forms of nucleotides, with a net ATPase activity. ATPases typically use the energy of ATP hydrolysis to alter intra- or intermolecular protein:protein interactions. We currently hypothesize that the Nm23-ATPase activity may permit Nm23 to bind/unbind other proteins in a chaperone function,

and that this activity would be regulated in the signal transduction process. The potential relevance of the pathway identified in my laboratory to the biological function of *nm23* was tested in a series of control and *nm23-1* transfected murine melanoma cell lines. Successive passages of each cell line were (1) injected into mice to confirm metastatic potential; (2) [<sup>32</sup>P] orthophosphate labeled to determine *in vivo* Nm23-phosphoserine levels; (3) assayed for NDPK activity and (4) tested for exogenous and endogenous *nm23* RNA levels. We observed a quantitative increase in the *nm23-1* transfectants in *nm23* RNA levels, metastasis suppressive ability and Nm23-phosphoserine levels; no difference was found in the NDPK activity of the control- and *nm23-1* transfectants. The data support the hypothesis that the serine phosphorylation pathway of Nm23 may be relevant to its biological functions.

While the laboratory will pursue the elevation of Nm23 expression as a translational approach, a second application is also under investigation. Control and *nm23* transfectants of murine melanoma, human MDA-MB-435 breast carcinoma and human OVCAR-3 ovarian carcinoma cell lines have been tested for the growth inhibiting activity of cisplatin *in vitro*. The *nm23* transfectants have been more sensitive to cisplatin in all cases. Injection of the murine melanoma cell lines *i.v.* has been used to test the cisplatin sensitivity of these lines *in vivo*. Delivery of a single injection of cisplatin *i.v.* two-three days after *i.v.* injection of tumor cells suppressed pulmonary metastasis development by the *nm23* transfectants to a significantly greater degree than the control transfectants. Thus, for one system, *in vivo* data has confirmed *in vitro* data. The mechanism of increased cisplatin sensitivity is under investigation. In preliminary experiments, we have observed no difference in cisplatin-induced apoptosis among the control- and *nm23* transfected cell lines. In collaboration with Dr. Wilhelm Bohr, NIA, we are investigating cisplatin induced DNA adduct formation and repair. Preliminary data suggest that in the MDA-MB-435 breast carcinoma cell lines, cisplatin forms greater interstrand DNA adducts in the *nm23-H1* transfectants. These data, if confirmed and extended, suggest a translational approach to use Nm23 expression to improve the efficacy of chemotherapy.

#### Publications:

Lakso M, Steeg PS, Westphal H. Embryonic expression of *nm23* during mouse organogenesis. *Cell Growth and Differentiation* 1992;3:873-9.

Leone A, Seeger RC, Arboleda MJ, Hong CM, Hu YY, Stram D, Brodeur G, Slamon DJ, Steeg PS. Evidence for *nm23* RNA overexpression, DNA amplification and mutation in aggressive childhood neuroblastomas. *Oncogene* 1993;9:855-65.

Steeg PS. Suppressor genes in breast cancer: An overview. In: Lippman M, Dixon R, eds. *Genes, oncogenes and hormones in breast cancer*. Boston: Kluwer Academic Publishers, 1992:45-57.

Steeg PS. Invasion and metastasis. *Current Opinion in Oncology* 1992;4:134-41.

MacDonald NJ, Steeg PS. Molecular basis of tumor metastasis. *Cancer Surveys* (in press)



Steeg PS, De La Rosa A, Flatow U, MacDonald NJ, Benedict M, Leone A. Nm23 and breast cancer metastasis. Breast Cancer Research Treatment (in press)

Leone A, Flatow U, Van Houtte K, Steeg PS. Transfection of the human nm23-H1 into the human MDA-MB-435 breast carcinoma cell line: Effects on colonization, metastatic potential and enzymatic activity. Oncogene (in press)

Kantor JD, McCormick B, Steeg PS, Zetter BR. Inhibition of cell motility after nm23 transfection of human and murine tumor cells. Cancer Res (in press)

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

PROJECT NUMBER

Z01 CB 09163-06 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Signal Transduction Therapy--Basic Science

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

PI:	E. Kohn	Senior Staff Fellow	LP NCI
OTHER:	W. Jacobs	General Fellow	LP NCI
	K. Cole	General Fellow	LP NCI
	Y. Kim	Visiting Associate	LP NCI
	R. Alessandro	Visiting Fellow	LP NCI
	M. Soltis	Stay-in-School	LP NCI
	L. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS (if any)

Medicine Branch, DCT, NCI; K. Rodland, Ph.D., Oregon Health Sciences University;  
 C. Felder, Ph.D., LCB, NIMH; D. Volpe, Ph.D., CDER, FDA

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

1.2

OTHER:

1.3

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

D

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

Our investigation of signal transduction pathways and their relationship to cancer has grown in many ways over the last year. We have used CAI as a tool to further our studies of key Ca<sup>++</sup>-mediated pathways. The molecular dissection of CAI demonstrated that CAI inhibition of Ca<sup>++</sup>-mediated signaling events is concordant with inhibition of proliferation. Novel compounds were identified and characterized and a patent application was filed. Further studies have identified a role for Ca<sup>++</sup> influx regulation of tyrosine phosphorylation of phospholipase C- $\gamma$  which produces inositol trisphosphate and internal calcium release. Use of CAI helped clarify that receptor-operated Ca<sup>++</sup> influx mediated maintenance of the malignant phenotype of CHO cells.

Biotinylated CAI (B-CAI) has been produced. Avidin-linked B-CAI and a CAI analog have been used to immunize rabbits. B-CAI and/or antisera will be used for expression cloning of the CAI binding sites and studies at the protein level. Subtraction analysis of gene products amplified in cells resistant to chronic CAI exposure has identified several candidate genes. Transcripts of 1.2 and 2.7 kb were expressed in A2058 resistant to 10, 20, and 30  $\mu$ M CAI and not in wild type A2058. Clones identified using the cDNA subtraction probe are being characterized. CAI has been used as a tool to investigate Ca<sup>++</sup>-regulated gene expression. CAI inhibits IL-2 gene transcription through several transactivating proteins. It also stimulates and inhibits Ca<sup>++</sup> and growth factor-mediated production of a viral enhancer, VL-30.

We have shown that CAI is antiangiogenic *in vitro* and *in vivo*. Incubation of human umbilical vein endothelial cells with CAI inhibited proliferation, adhesion, motility, MMP-2 production, and tube formation, and CAI inhibited microvascular proliferation in chick chorioallantoic membrane assays. Studies are ongoing to identify the sites of this Ca<sup>++</sup>- mediated signal transduction inhibition of angiogenesis.

Major Findings:

1. Concordance between inhibition of calcium signaling and proliferation. Chemical modifications of the CAI structure have been produced and tested. The effects on proliferation and signal transduction were concordant, supporting the hypothesis that the mechanism of action of this drug is through inhibition of the selected signal transduction pathways. Selected active analogs will be evaluated for clinical potential. A patent application has been filed to cover new matter and new use.

Further investigation of signaling pathways regulated by CAI has identified calcium and CAI-dependent tyrosine phosphorylation of phospholipase C-gamma through the muscarinic receptor. This demonstrated calcium (and CAI)-dependent and -independent pools of inositol phosphates. Further studies will expand this observation with other ligand-directed tyrosine phosphorylation pathways.

2. Characterization of resistance to inhibition of calcium signal transduction. We have developed A2058, OVCAR3, and CHOm5 cell sublines which grow in the continuous presence of CAI. To date, A2058 cells resistant to up to 40  $\mu\text{M}$  CAI by continuous exposure have been generated. Characterization is proceeding along two lines: molecular and biologic. Subtraction cDNA analysis of A2058 cells resistant to 20  $\mu\text{M}$  CAI (A2058-20R) against wild type A2058 cells (WT) has demonstrated selectively augmented transcripts of 1.2 and 2.7 kb, in the 10R, 20R, and 30R cells by Northern blot analysis. Library screening is underway and candidate clones are being studied. The biologic analysis will include evaluation of doubling time, MMP-2 production, migration, signal transduction parameters, and clonigenicity in soft agar and tumorigenicity in nude mice.

3. Signal transduction regulation of angiogenesis. Calcium-mediated events have been shown to be important in the migration, adhesion, and proliferation of normal and malignant cells. We have demonstrated that CAI inhibits human umbilical vein endothelial cell adhesion, motility, proliferation, and MMP-2 production in the same effective concentration range as tumor cells. Preliminary results indicate that CAI inhibits chick chorioallantoic membrane angiogenesis. Ongoing investigations focus on the efficacy of molecular analogs of CAI and pursue CAI inhibition of bFGF-mediated signal transduction events in the HUVEC.

4. Identification and cloning of CAI binding site. We have approached several ways. We have evaluated biotinylated CAI for direct binding to electrophoresed immobilized proteins and have made CAI-affinity columns. CAI and analogs are being linked either via biotin/avidin or directly to antigenic haptens for production of antibody. Preliminary experiments using CAI-affinity columns suggest specific binding of several proteins. Conditions are being worked out to characterize and purify these proteins. Analysis of biotinyl-CAI and antiserum preparations will provide evidence that these may be used for expression cloning of the binding site. If directed reagents are not forthcoming, functional expression cloning using calcium imaging may be used for binding site identification.

5. Effect of calcium-mediated signaling on gene transcription. Two different collaborations have been set up to investigate the effect of CAI on gene transcription, in response to the observations that CAI inhibits expression of MMP-2 from human A2058 melanoma cells. In collaboration with Randall Kincaid, Ph.D. (NIAAAA), we have demonstrated that CAI inhibits transcription of several independent gene regulatory proteins (AP-1, NFkB, OCT-1) in a dose dependent fashion. These transcription factors are regulated by calcium and calcineurin. The effective concentration range was identical to that which inhibits tumor cell growth, signaling, and migration. A paradoxical effect of time dependent stimulation or inhibition of production of the VL-30 enhancer has been shown in collaboration with Karin Rodland, Ph.D. (Oregon Health Science University). This enhancer is regulated by intracellular calcium concentrations and growth factor stimulation of calcium mobilization.

6. Effects of signal transduction therapy on hematopoiesis. Collaboration with Donna Volpe, Ph.D. (FDA) demonstrated that CAI inhibits cloning efficiency of murine CFU-GM and BFU-E *in vitro* and when bone marrow cells from CAI-treated mice are placed in culture. The IC<sub>50</sub> concentrations were in the range of 0.1-1.0 µg/ml for both lineages. The extent of CAI exposure (AUC) correlated with the extent of inhibition of colony formation, independent of time of exposure, from 2 hours to continuous exposure. Human bone marrow samples processed under similar conditions demonstrated dose dependent inhibition of colony formation, but with higher IC<sub>50</sub> values. Results with the molecular analogs of CAI are pending and the effect of CAI on hematopoietic growth factor-stimulated calcium mobilization is being studied.

#### Publications:

Kohn EC, Liotta LA. Therapeutic application of an anti-invasion compound. (issued 21 July 1992; #5,132,315)

Kohn EC, Sandeen, MA, Liotta LA. *In vivo* efficacy of a novel inhibitor of selected signal transduction pathways including calcium, arachidonate, and inositol phosphates. *Cancer Res* 1992;52:3208-12.

Felder CC, Ma AL, MacArthur L, Kohn EC. Tumor suppressor function of muscarinic acetylcholine receptors in CHO cells. *Proc Natl Acad Sci USA* 1993;90:1706-10.

Gusovsky F, Lueders JE, Kohn EC, Felder CC. Muscarinic receptor-mediated tyrosine phosphorylation of phospholipase C-γ: An alternative mechanism for cholinergic-induced phosphoinositide breakdown. *J Biol Chem* 1993;268:7768-72.

Kohn EC, Liotta LA. Invasion and metastasis: an old problem with new approaches. *Oncology* 1993;7:47-52.

Kohn EC. Aging issues in invasion and metastasis: Fertile ground for investigation. *Cancer* 1993;71:552-7.

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

PROJECT NUMBER

Z01 CB 09164-06 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Stetler-Stevenson	Medical Officer	LP NCI
OTHER:	D. Kleiner	Expert	LP NCI
	A. Levy	Microbiologist	LP NCI
	A. Murphy	Staff Fellow	LP NCI
	O. Malykh	Special 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 STAFF YEARS:

4.0

PROFESSIONAL:

3.0

OTHER:

1.0

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

B

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

In order to investigate the role of matrix metalloproteinases (MMP) in tumor invasion and metastases, we have focused on the multilevel regulation of these enzymes. Studies have shown that in contrast with other members of the MMP enzyme family, 72 kDa gelatinase A levels are increased in response to TGFβ1, are unaffected by the tumor promoting phorbol esters, and show elevated levels in colorectal, breast, thyroid, ovarian and bladder tumor tissues when compared with adjacent normal mucosa tissues. We have identified a cellular activation mechanism which is cell surface associated and specific for the 72 kDa gelatinase A enzyme, and which can be induced by pretreatment with phorbol esters or concanavalin A. This cellular activation mechanism does not affect other members of the collagenase gene family. This activation mechanism appears to require cell surface binding of the gelatinase A enzyme, and we have identified a putative gelatinase A receptor.

We have studied the structure of the latent enzyme TIMP-2 complex through production of enzyme deletion mutants and enzyme inhibitor cross linking studies. These studies demonstrate that the 72 kDa gelatinase A has at least two TIMP-2 binding domains. The principal binding domain is located in the C-terminal, hemopexin-like domain of the enzyme. This binding site is available in the latent enzyme form. The second binding site is at the enzyme active site and only becomes available following organomercurial mediated enzyme activation.

Finally, antipeptide antibodies against the 92 kDa gelatinase B, interstitial collagenase, stromelysin-1 and stromelysin-2 have been prepared and characterized.

Major Findings:

1. The 72 kDa gelatinase A enzyme is activated by organomercurial compounds *in vitro*.
2. There is specific cellular activation mechanism *in vivo* that require cell surface binding of latent enzyme inhibitor complex.
3. Activation subsequently results in removal of an 80 amino acid profragment peptide both *in vitro* and *in vivo*.
4. The 80 amino acid profragment contains a highly conserved peptide region which is responsible for maintaining the latency of the proenzyme through a sulfhydryl-metal atom interaction as determined by titration studies of the free sulfhydryls associated with the holoproenzyme and apoproenzyme preparations.
5. Synthetic peptides containing the highly conserved region from the amino terminal profragment of the 72 kDa gelatinase A inhibit enzyme proteolytic activity against gelatin and type IV collagen *in vitro*. In addition, these peptides specifically block tumor cell invasion across reconstituted basement membranes *in vitro*.
6. The cellular activation mechanism for the 72 kDa gelatinase A is cell surface associated; inhibited by metalloproteinase inhibitors; specific for the 72 kDa enzyme; induced by specific treatments in both primary cell culture and metastatic human tumor cell lines.
7. We have performed preliminary characterization of a cell surface receptor for the gelatinase A/TIMP-2 complex. This receptor has a  $K_d = 2.0 \times 10^{-9}M$  and approximately 50,000 sites/cell.
8. The 72 kDa type IV collagenase is secreted as a complex with TIMP-2 in the presence of excess free TIMP-2.
9. 72 kDa gelatinase A has two TIMP-2 binding sites. These are located in the C-terminal hemopexin-like domain and the enzyme active site.

Publications:

Kleiner DE Jr, Unsworth EJ, Krutzsch HC, Stetler-Stevenson WG. Higher-order complex formation between the 72-kilodalton type IV collagenase and tissue inhibitor of metalloproteinases-2. *Biochemistry* 1992;31:1665-72.

Campo E, Merino MJ, Liotta LA, Neumann R, Stetler-Stevenson WG. Distribution of the 72 kDa type IV collagenase in non-neoplastic and neoplastic thyroid tissue. *Hum Pathol* 1992;23:1395-1401.

Campo E, Merino MJ, Tavassoli FA, Charonis AS, Stetler-Stevenson WG, Liotta LA. Evaluation of basement membrane components and the 72 kDa type IV collagenase in serous tumors of the ovary. *Am J Surg Pathol* 1992;16:500-7.

Melchiori A, Albini A, Ray SM, Stetler-Stevenson WG. Inhibition of tumor cell invasion by a highly conserved peptide sequence from the matrix metalloproteinase enzyme prosegment. *Cancer Res* 1992;52:2353-6.

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Sreenath T, Matrisian LM, Stetler-Stevenson WG, Galtoni-Celli S, Pozzatti RO. Expression of matrix metalloproteinase genes in transformed rat cell lines of high and low metastatic potential. *Cancer Res* 1992;52:4942-7.

Fridman R, Fuerst TR, Bird RE, Höyhty M, Oelkuck M, Kraus S, Komarek D, Liotta LA, Berman ML, Stetler-Stevenson WG. Domain structure of human 72 kDa gelatinase/type IV collagenase: Characterization of proteolytic activity and identification of the tissue inhibitor of metalloproteinase-2 (TIMP-2) binding regions. *J Biol Chem* 1992;267:15398-15405.

Emonard HP, Remacle AG, Noël AC, Grimaud J-A, Stetler-Stevenson WG, Foidart J-M. Tumor cell surface-associated binding site for the M<sub>r</sub> 72,000 type IV collagenase. *Cancer Res* 1992;52:5845-8.

Fridman R, Bird RE, Höyhty M, Oelkuck M, Komarek D, Liang C-M, Berman ML, Liotta LA, Stetler-Stevenson WG, Fuerst TR. Expression of human recombinant 72 kDa gelatinase and TIMP-2: Characterization of complex and free enzyme. *Biochem J* 1993;289:411-16.

Brown PD, Kleiner DE, Unsworth EJ, Stetler-Stevenson WG. Cellular activation of 72-kDa type IV procollagenase/TIMP-2 complex. *Kidney Int* 1993;43:163-70.

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

PROJECT NUMBER

201 CB 09179-05 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Stetler-Stevenson	Medical Officer	LP NCI
OTHER:	J. Ray	Staff Fellow	LP NCI
	A. Murphy	Staff Fellow	LP NCI
	G. D'Orazi	Visiting Fellow	LP NCI
	D. Kleiner	Expert	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 STAFF YEARS:

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PROFESSIONAL:

3.0

OTHER:

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 (a1) Minors  
 (a2) Interviews

B

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

We have isolated and characterized the complete primary structure of a new member of the tissue inhibitor of metalloproteinase family (TIMP family) which we refer to as TIMP-2. Recent studies have shown that all cells studied to date which secrete the 72 kDa type IV collagenase enzyme secrete this enzyme as a complex with TIMP-2. The majority >95% of secreted 72 kDa gelatinase is found in complexed form as most cells tested secrete 2-4 fold as free TIMP-2. Our studies have shown that TIMP-2 transcription is regulated independently of both TIMP-1 and the 72 kDa type IV collagenase enzyme. We have also demonstrated that TIMP-2 is anti-angiogenic. The mechanism for this effect is two fold; through inhibition of endothelial cell proliferation and blocking endothelial cell-mediated matrix proteolysis. TIMP-2 inhibits tumor cell invasion through reconstituted basement membranes *in vitro*, and this inhibitor demonstrates erythroid potentiating activity (EPA). TIMP-2 inhibits proteolytic opening of the blood brain barrier in hemorrhagic stroke models.

TIMP-2 genomic clones have been obtained and partial sequencing has identified two introns in the 3' end of the gene. The gene appears to be single copy and is localized on human chromosome 17q25.

We have examined the TIMP-2 protein structure and have localized the metalloprotease inhibitory domain to the N-terminal half of the molecule. Further sublocalization has been attempted using a synthetic peptide approach as well as protein crystallization for x-ray diffraction and NMR-spectroscopy.



Major Findings:

1. There is a novel 21 kDa protein which binds selectively and with 1:1 molar stoichiometry to the latent form of the human 72 kDa type IV collagenase to form a proenzyme inhibitor complex.
2. All cells which produce the 72 kDa type IV collagenase complex produce this enzyme in complexed form with excess free TIMP-2 present.
3. Studies of the transcription of TIMP-2 mRNA reveal that TIMP-2 is regulated independently from TIMP-1 and the 72 kDa type IV collagenase.
4. TIMP-2 inhibits tumor cell invasion through a reconstituted basement membrane *in vitro*.
5. TIMP-2 inhibits angiogenesis in the chick chorioallantoic membrane assay and this effect is due to inhibition of endothelial cell proliferation and antiprotease activity.
6. TIMP-2 has erythroid-potentiating activity.
7. The human TIMP-2 gene is single copy and localized to chromosome 17q25. The gene structure contains two introns in the 5' portion.
8. There are two TIMP-2 binding sites on the human 72 kDa type IV collagenase. The C-terminal binding site does not interfere with enzyme activation or substrate binding.
9. The protease inhibitory domain of TIMP-2 is located in the N-terminal half of the TIMP-2 molecule.

Publications:

Seftor REB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC. Role of the  $\alpha_v\beta_3$  integrin in human melanoma cell invasion. Proc Natl Acad Sci USA 1992;89:1557-61.

Kleiner DE Jr, Unsworth EJ, Krutzsch HC, Stetler-Stevenson WG. Higher-order complex formation between the 72-kilodalton type IV collagenase and tissue inhibitor of metalloproteinases-2. Biochemistry 1992;31:1665-72.

Rosenberg GA, Kornfeld M, Estrada E, Kelley RO, Liotta LA, Stetler-Stevenson WG. TIMP-2 blocks proteolytic opening of blood-brain barrier by type IV collagenase. Brain Res 1992;576:203-7.

Stetler-Stevenson WG, Bersch N, Golde DW. Tissue inhibitor of metalloproteinase-2 (TIMP-2) has erythroid-potentiating activity. FEBS Lett 1992;296:231-4.

Stetler-Stevenson WG, Liotta LA, Seldin MF. Linkage analysis demonstrates that the *Timp-2* locus is on mouse chromosome 11. *Genomics* 1992;14:828-9.

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

PROJECT NUMBER

Z01 CB 09185-04 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

**G Proteins and Tumor Cell Motility**

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

PI:	S. Aznavoorian	Biotechnology Fellow	LP NCI
OTHER:	L. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI
	D. Savarese	Clinical Associate	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 STAFF YEARS:

1.1

PROFESSIONAL:

1.1

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 (a1) Minors  
 (a2) Interviews

B

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

The amoeboid migration of tumor cells is characterized by protrusion of pseudopodia. These specialized regions of the cell cortex thus constitute the basic machinery of cell locomotion. Type IV collagen stimulates chemotaxis of human melanoma cells through a G protein-coupled receptor; however, the downstream effectors of G protein activation which ultimately cause pseudopodial protrusion and cell translocation are unknown. We have isolated chemo-attractant-induced pseudopodia from 1  $\mu$ m-pore chemotaxis filters after mechanical shearing from the tumor cell bodies. Analysis by Western immunoblot has revealed that the pseudopodial extracts have an increased ratio of G $\alpha$ 2 to G $\alpha$ o, relative to lysates from unstimulated whole cells. Thus, it appears that G $\alpha$ o is sequestered out of pseudopods, and may not be involved in transducing motility signals. Western immunoblotting has confirmed the presence of actin and the 280 kD actin binding protein in pseudopodial extracts, supporting the premise that pseudopodia contain actin cross-linked into a three dimensional, orthogonal network. A micropipette system was used to follow the dynamics of pseudopodial protrusion in an individual tumor cell stimulated by type IV collagen, with and without an inhibitor of signal transduction (PT). In untreated cells, type IV collagen induced extension of irregularly-shaped pseudopodia. Treatment of cells with PT, which inhibits G protein-linked signals but not the type IV collagen-induced Ca<sup>2+</sup> burst, inhibited extension of the pseudopod while allowing formation of a much smaller, symmetrical membrane bleb. Conceivably this is due to Ca<sup>2+</sup>-activated actin disassembly and osmotic fluid flux. Thus, two stages of pseudopod protrusion are revealed which are mediated by separate intracellular signals. Goals of future studies are to identify the signals and effector proteins regulating pseudopodial protrusion and cell motility.

Major Findings:

Directed tumor cell migration requires the coordinated action of many interrelated steps which are incompletely understood, including binding of attractant to its receptor, transduction of the signal(s), generation of intracellular messengers, and activation of effector enzymes/ion channels which initiate pseudopod protrusion and ultimately cell translocation. Chemotaxis of A2058 human melanoma cells to type IV collagen is transduced by a pertussis toxin (PT)-sensitive G protein; however, the biochemical pathway from activated G protein to pseudopodial protrusion and migration is unknown. Pseudopodial protrusion is central to tumor cell amoeboid chemotaxis, and work with phagocytic leukocytes has led to the belief that most of the "machinery" for cell motility is contained within this specialized area of the cell cortex. Consequently, a major goal of this project is to learn the biochemical mechanisms of chemoattractant-induced pseudopodia formation. Our study of tumor cell pseudopodia has proceeded along two parallel lines of investigation; in one set of studies, we have sought to identify motility-associated proteins within chemoattractant-induced pseudopodia, including specific receptors, transducers, effectors, and actin binding/regulatory proteins. This has required the isolation of pseudopodia in quantities large enough for immunoblot and biochemical analysis. We have accomplished this by use of filters in chemotaxis assays which are only 1  $\mu\text{m}$  in pore size, in contrast to the typical 8  $\mu\text{m}$  pores. Attractant-stimulated tumor cells extend pseudopods through these small pores, but cannot crawl through entirely. Tumor cell bodies are mechanically sheared off from their pseudopodia in the pores by scraping the filter along a rubber blade. Pseudopodial material is then collected by soaking the filter in Tris buffer containing protease inhibitors. Concentration of this material and analysis by Western immunoblot with G protein antibodies has revealed that pseudopod "extracts" are enriched in  $G_{1\alpha_2}$  relative to  $G_{\alpha_0}$ , when compared to lysates of whole, unstimulated cells. This would imply that  $G_{\alpha_0}$  is "sequestered" out of developing pseudopods, and perhaps not involved in transducing motility signals. It is of interest to determine if the ratio of  $G_{1\alpha_2}$  to  $G_{\alpha_0}$  varies among pseudopod preparations isolated with different attractants. This type of analysis is possible with A2058 cells, which migrate chemotactically to multiple ligands, some of which do not transduce their signals through such a G protein. Immunoblotting of pseudopod extracts has confirmed the presence of actin in these preparations, as well as a 280 kD actin binding protein (ABP). This protein cross-links actin filaments at right angles into orthogonal networks, the type of actin network found in peripheral cytoplasm of macrophages and *Dictyostelium* amoeba, two other types of motile cells. The importance of the 280 kD ABP in directed cell migration has been demonstrated in another line of human melanoma cells; therefore, its identification and possible enrichment in A2058 pseudopodia implicates its involvement in chemotaxis of these cells as well. Ultimately, we need to learn how the expression, distribution, and/or activation of these crucial motility proteins are regulated by chemoattractant stimulation.

To analyze the stages of formation of individual pseudopodia, we have used a micropipette system to investigate the dynamics of pseudopodial protrusion in a single attractant-stimulated cell. Using type IV collagen as attractant, we observed continuous extension of pseudopodia into the micropipette over a period of >40 minutes. Pretreatment of cells with PT, which abolishes chemotaxis by uncoupling the G protein from its receptor, blocked extension of pseudopodia into the pipette; however, these cells still formed a much smaller bleb, or outpouching of the membrane, which stopped growing at ~20 min. PT does not inhibit the type IV collagen-induced  $Ca^{+2}$  burst in these cells; therefore, we speculate that the bleb is the result of  $Ca^{+2}$ -mediated actin disassembly and local osmotic fluid flux into the region. This is the first demonstration of distinct "stages" of pseudopod formation, regulated by separate intracellular signals ( $Ca^{+2}$  and G proteins). Use of this dynamic, *in vivo* system with other attractants and inhibitors of different stages of signalling should eventually yield information as to where the distinct chemoattractant-stimulated pathways merge to cause pseudopodial protrusion and cell migration. Knowledge gained from study of the mechanisms of motility at this level should ultimately lead to strategies to prevent the transition from *in situ* to invasive carcinoma, in which motility plays a role.

#### Publications:

Aznavoorian S, Murphy AN, Stetler-Stevenson WG, Liotta LA. Molecular aspects of tumor cell invasion and metastasis. *Cancer* 1993;71:1368-83.

Savarese DMF, Russell JT, Fatatis A, Liotta LA. Type IV collagen stimulates an increase in intracellular calcium: Potential role in tumor cell motility. *J Biol Chem* 1992;267:21928-35.

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

PROJECT NUMBER

Z01 CB 09352-03 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

AAMP-1, A T-Cell Activation Protein with a Heparin and Cell Binding Site

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

PI: M. Beckner Biotechnology Fellow LP NCI  
 OTHER: L. Liotta Chief, Tumor Invasion and Metastases Section LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.25

PROFESSIONAL:

1.00

OTHER:

0.25

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

B

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

A human cDNA clone has been isolated that encodes a new immunoglobulin superfamily member, AAMP. Its sequence predicts a novel 45.7 kDa protein with a potential transmembrane domain, immunoglobulin domains, and a heparin binding consensus sequence near the amino terminus. Anti-peptide antibodies, created to react with specific regions of AAMP's predicted sequence, react positively with recombinant AAMP protein thus confirming its predicted sequence. The immunoglobulin-like domains of AAMP are homologous with multiple domains from immunoglobulin superfamily members, including the "deleted in colon carcinoma" (DCC) protein, neural cell adhesion molecule (NCAM) and intercellular cell adhesion molecule (ICAM). Single, 1.6 kilobase mRNA transcripts of AAMP are present in many adult and fetal tissues. Studies of the RNA message levels in T cell activation, a known modulating event for adhesive proteins, showed marked augmentation of AAMP with peak levels at 24 hours. A peptide (P189), derived from the predicted heparin binding domain of AAMP, binds heparin and elicits heparin dependent cell aggregation. Substituted or scrambled forms of this peptide could not induce comparable human melanoma cell aggregation. Immobilized P189 and its variants that contained the heparin binding consensus sequence mediated cell attachment significantly more than the variants that lacked the consensus sequence. Heparin abolished the cell attachment activity of P189. AAMP can potentially mediate cell-cell and cell-matrix interactions.

An anti-peptide, polyclonal antibody specific for P189 reacts with a protein that is comparable in size to AAMP in human brain tissue and in activated T lymphocytes. When aliquots of cell lysates from a time course of T cell activation are standardized either according to cell number or amount of protein, the expression of the 56 kDa anti-P189 specific protein increases after 72 hours.

Future Plans:

1. AAMP recombinant protein will be purified for functional studies in tumor cells, T lymphocytes, and other benign cells (heparin/cell binding, extracellular matrix adhesion, motility inhibition/stimulation, etc.) and for polyclonal antibody production.
2. Additional polyclonal antibodies specific for the AAMP recombinant protein, antigenic peptides derived from AAMP's sequence, and (eventually) purified AAMP from mammalian tissue will be produced. These will be helpful in further confirmation of AAMP's identification, distribution in tissues, and cell localization. They will also be used in the functional studies mentioned above.
3. AAMP protein from mammalian tissue will be purified for further confirmation of its sequence, determination of the amino terminus, and detection of post-translational modifications. The 56 kDa protein in brain tissue (also in calf brain) will be the candidate protein for purification.
4. Samples of human tissue from malignancies with the benign counterparts has been processed for total RNA isolation and will be tested with AAMP hybridization studies to determine AAMP's relative mRNA amounts in malignant tissues compared to related benign tissues. Protein expression also needs to be evaluated. The diagnostic usefulness of AAMP in distinguishing reactive from malignant cells will be determined.
5. The effects of AAMP expression on T cells, macrophages, and stromal cells and on their interactions with tumors and their benign counterparts (when available) will be determined.
6. The peptide, P189, derived from AAMP's amino terminal region, that forms particulates and binds either cells or heparin will be further evaluated for its medical usefulness as a heparin localizing agent.
7. The chromosomal localization of AAMP and its genomic sequence will be studied. Information regarding its regulation can be obtained.
8. Anti-peptide antibodies specific for the sequence of the protein purified with the monoclonal antibody, 1AA3, will be tested for functional effects on cell adhesion and motility. Hopefully this protein can be further characterized with anti-peptide antibodies on immunoblots and the gene cloned from an expression library.

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

PROJECT NUMBER

Z01 CB 09374-01 LP

PERIOD COVERED

Novel Signaling Pathways in A2058 Cells

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

October 1, 1992 to September 30, 1993

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

PI:	E. Kohn	Senior Staff Fellow	LP NCI
OTHER:	W. Jacobs	General Fellow	LP NCI
	Y. Kim	Visiting Associate	LP NCI

COOPERATING UNITS (if any)

Christian Felder, Ph.D., Laboratory of Cell Biology, NIMH

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.7

PROFESSIONAL:

0.2

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 unrounded type. Do not exceed the space provided.)

Novel calcium-sensitive ligand-receptor interactions have been sought for further investigation of calcium-mediated signal regulation. The A2058 human melanoma cell line has been chosen. Calcium-influx responses have been documented when these cells are bathed with carbachol, the muscarinic acetylcholine agonist, ATP, agonist of the P2Y receptors, and thrombin. The observations of the carbachol-induced activity have been characterized and have demonstrated that there is a low abundance, high affinity muscarinic receptor on in the membrane of A2058 cells. When activated, this receptor stimulates calcium influx and intracellular release, release of arachidonic acid, and inhibition of cAMP production. No generation of inositol phosphates has been demonstrated using three different assay systems. Biologic behavior for this receptor has been demonstrated by its inhibitory action on the soft agar colonization of A2058 cells in the presence of carbachol which is completely abrogated by coincubated with atropine. Molecular characterization of this receptor suggests that it may have similarity to the m2 subtype by Northern analysis and the m3 subtype by pharmacologic analysis. PCR and detailed pharmacologic mapping are ongoing.



Major Findings:

1. Identification of alternative calcium-mobilizing ligands in A2058 cells. Using single cell fluorescent photometry, several new calcium-mobilizing ligands have been identified for the A2058 human melanoma cell line. These include carbachol, the stable acetylcholine agonist which stimulates muscarinic receptors, ATP, agonist of P2Y receptors, and thrombin. In addition to the work of Savarese et al with type IV collagen, this brings to 4 the number of potential signal transduction agonists for investigation. Further characterization of the carbachol response has been accomplished.

2. Identification of endogenous muscarinic receptors (mAChR) on the A2058 human melanoma cell. A functional mAChR has been demonstrated by its ability to stimulate calcium internal release and influx. Further studies have demonstrate that this receptor may also stimulate arachidonic acid release. Surprisingly, no receptor-stimulated generation of inositol phosphates can be demonstrated, as would be expected by the odd-numbered receptor pattern of signaling seen. Activation of this receptor inhibited forskolin-stimulation of cAMP production, a function of even-numbered muscarinic receptors. These data suggest that either this receptor is a novel biochemical hybrid of even and odd-receptor function or that there are two classes of receptors on these cells. Biologic function of this receptor has been observed. Addition of carbachol to soft agar colonization experiments resulted in inhibition of colony formation. This inhibition was reversible by the muscarinic antagonist, atropine.

3. Molecular and pharmacologic characterization of the novel muscarinic receptor. Northern blot analysis of A2058 polyadenylated mRNA suggested that this receptor may be genetically related to the m2 subtype but not the m1, m3, m4, or m5 receptor subtypes under stringent hybridization conditions using selective riboprobes of the third cytoplasmic loop of the m5 receptor. PCR amplification of A2058 mRNA using oligonucleotides containing coding sequence from the highly conserved m5 muscarinic receptor transmembrane domains yielded fragments of expected size. Further amplification is being used to produce a large fragment for library screen and for direct sequencing. Receptor binding analysis has demonstrated 40 fmol receptor/mg A2058 membrane protein. Pharmacologic subtype analysis using subtype selective antagonists is under way.

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

PROJECT NUMBER

201 CB 09375-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Signal Transduction Therapy--Clinical

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

PI:	E. Kohn	Senior Staff Fellow	LP NCI
OTHER:	K. Cole	General Fellow	LP NCI
	L. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS (if any)

Cooperating Units: Eddie Reed, MD, Patricia Davis, RN, Medicine Branch, DCT, NCI; Developmental Therapeutics Program, DCT, NCI; Cancer Therapy Evaluation Program, DCT, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.7

PROFESSIONAL:

0.3

OTHER:

0.4

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 (a1) Minors  
 (a2) Interviews

A

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

The Investigational New Drug application to the FDA for oral administration of CAI to patients with refractory solid tumors was approved in January 1992 and the Phase I clinical trial began accrual in March 1992. The protocol is run through and in collaboration with the Medicine Branch, DCT, NCI. Eligible patients receive a test dose of CAI in PEG-400 solution followed by blood sampling for pharmacokinetic analysis over one week. The following week, every other day administration of CAI begins with frequent blood sampling to measure plasma levels. Since initiation, 15 patients have received CAI on protocol. In all cases, CAI blood levels have been readily measured in the plasma of patients and in the pleural fluid of one patient. The plateau blood levels ranged from 2  $\mu\text{M}$  to over 10  $\mu\text{M}$  over the three dose escalation levels. Calculated pharmacokinetics suggest a long half life at all administered doses with increasing AUC also seen with increasing dose. Toxicity has been minimal. Mild nausea with rare vomiting and mild mood changes appear to be drug-related and may be related to peak dose. One episode of peripheral neuropathy and one episode of neutropenia have been observed; both appear to be related to the total drug exposure. Gelcap formulation is now available and test doses with pharmacokinetics will evaluate the bioavailability, toxicity, and efficacy of this new formulation. Three patients have had progression of disease during the first 28 day period of CAI administration. The remaining patients have had >25% increase in disease, measurable stability, or reductions of up to 22% in their disease. Further dose escalation is ongoing and Phase II trials are planned.

Major Findings:

1. Demonstration of CAI in plasma of patients (previously under Z01 CB 09163-05 LP). An accurate, specific, reliable, and sensitive high performance liquid chromatography assay to measure CAI in plasma was developed and verified. A patent application has been submitted for this method. This method has been used to support pharmacokinetic analysis of CAI in the patients on the clinical trial and for myelosuppression studies underway in collaboration with the FDA. A measurable CAI level was demonstrated in the pleural fluid of one patient who developed a malignant effusion.

2. Pharmacokinetic analysis of CAI. Since initiation of the clinical trial in March of 1992, 15 patients have been treated on three dose escalation levels with orally administered CAI. Measurable blood levels have been demonstrated by HPLC analysis in all patients. Initial test doses have been done for pharmacokinetic analysis and pharmacodynamic correlation to toxicity and efficacy which may be demonstrated. The estimated half-life of CAI is long, ranging from 24-60 hrs in patients receiving 100 mg/m<sup>2</sup>/d to 60-100 hrs in patients receiving level 3, 330 mg/m<sup>2</sup> every other day. Drug exposure, area under the curve, has gone up accordingly. Peak/plateau levels of CAI at the end of the 28 day dosing period has reached 10 µM in several patients. This is the level that we target *in vitro* for inhibition of signal transduction and proliferation. Chromatograms of HPLC measurements of CAI have suggested early peaks which change in response to increasing exposure to CAI, indicative of metabolite production. We are collaborating with Jerry Collins, Ph.D. of the FDA to further identify these metabolites and the metabolic pathways involved. Preliminary evidence suggests that the P450 hepatic metabolism pathway is involved.

3. Toxicity profile of CAI. Dose limiting toxicity of orally administered CAI has not been reached. The most common complaint of the patients is the taste and texture of the PEG-400 solution. Only mild nausea and minimal mood depression has been seen and is likely to be drug related. These effects appear to be associated with the peak level after administration, 4 - 24 hours. Neutropenia (grade 4) and mild to moderate sensory stocking-glove neuropathy have been seen in one patient each. Neutropenia resolved within one week of discontinuation of CAI and the neuropathy continued to improve over 3 weeks off drug. Electromyographic analysis of the neuropathy is ongoing. Further dose escalation and pharmacokinetic analysis is planned.

4. Gelcap formulation. Our colleagues at the Developmental Therapeutics Program have developed and verified a gelcap formulation of CAI. Gelcaps have been produced with 15, 25, and 50 mg of CAI each. Test doses of both the gelcap and liquid formulations will be done for all patients on the next dose level to investigate the bioavailability and pharmacokinetics of the gelcap formulation. Micronized powder CAI in capsule or tablet is under development.

Publications:

Holmes KA, Chaffins C, Osborne B, Liotta LA, Kohn EC. Quantitation of plasma levels of a novel anticancer drug, carboxyamido-triazole (CAI) by high pressure liquid chromatography. J Chromatogr Biomed Appl (in press)

Kohn EC, Liotta LA. A detection and quantitation method for therapeutic agents in blood. U.S. Patent Application

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

PROJECT NUMBER

Z01 CB 09376-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Molecular Pathology of *c-erb-B-2* Oncogene Expression

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

PI:	P. Steeg	Research Biologist	LP NCI
OTHER:	U. Flatow	Biologist	LP NCI
	A. Warren	Guest Researcher	LP NCI

COOPERATING UNITS (if any)

Dennis Slamon, UCLA School of Medicine

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

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 (a1) Minors  
 (a2) Interviews

B

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

Overexpression of the *c-erb-2* oncogene (*Her-2/neu*) has been associated with poor clinical course in breast and ovarian cancers. *c-erb-B-2* encodes a growth factor receptor with 50% homology to the EGF receptor, and multiple ligands have been recently described. Experiments in progress are determining the effect of *c-erb-B-2* overexpression on the metastatic potential of human breast carcinoma cells.

Transfection experiments have been performed to overexpress *c-erb-B-2* in the human MDA-MB-435 breast carcinoma cell line at levels similar to those found in human tumors. Injection of control and *c-erb-B-2* transfected cells into the mammary fat pads of nude mice has resulted in equivalent rates of metastasis to the regional lymph nodes, but significant differences in distant metastases. The *c-erb-B-2* transfectants produced pulmonary metastases in 2-fold greater mice than did control transfectants. In contrast to the control transfected and parental cell lines, which typically contained one-several pulmonary metastases centered on capillaries, pathologic examination of lung sections from *c-erb-B-2* transfectants revealed tens-hundreds of metastases in a proportion of animals. This "3+" metastatic pattern was five-fold more prevalent in the *c-erb-B-2*, than the control transfectants. The metastatic lesions also infiltrated the parenchyma of the lung extensively. *In vitro* studies in progress will determine the effect of *c-erb-B-2* expression on cell growth, colonization, invasiveness and motility, both alone and in response to lung-derived factors and recombinant ligands. The experiments are expected to define a mechanism of action of the *c-erb-B-2* oncogene, and may lead to therapeutic strategies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
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PROJECT NUMBER

201 CB 09377-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Molecular Alterations in Premalignant and In Situ Breast Lesions

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

PI: P. Steeg Research Biologist LP NCI  
 OTHER: D. Weinstat-Saslow Staff Fellow LP NCI

COOPERATING UNITS (if any)

David Page, Vanderbilt University School of Medicine

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.7

PROFESSIONAL:

0.7

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.)

While the molecular alterations in infiltrating ductal breast carcinomas are well characterized, little is known about the molecular changes in early breast disease. We plan *in situ* hybridizations to determine the mRNA expression levels of cell cycle associated genes and growth factor receptor genes in low grade ductal carcinoma *in situ* (DCIS) and premalignant atypical ductal hyperplasias (ADH) of the human breast. The accuracy, sensitivity and reliability of *in situ* hybridizations using formalin fixed, paraffin embedded tumor sections has been determined for cyclins A and D, and the EGF receptor, FLG and BEK receptors for FGF, and IGF receptor. Subcloning of riboprobes for additional cyclin and receptor genes is underway.

In preliminary experiments, we have observed deregulated and relatively high levels of cyclin A in DCIS specimens. Cyclin A expression was homogenous within DCIS ducts and highest in relatively small lesions, indicating that it may be a very early change in carcinogenesis. These data stand in contrast to the cell cycle dependent expression of cyclin A at the G2-M boundary in many normal cells, and are consistent with a recent hypothesis that cyclin A may be an oncogene. Overexpression of cyclin D, which is normally expressed at the G1-S boundary of the cell cycle and is amplified in some infiltrating ductal carcinomas, has not been observed to date.

Our preliminary data indicate that low grade DCIS lesions express receptors for multiple growth factors. Consistent expression of the EGF receptor mRNA and FLG-FGF receptor mRNA have been observed. For both receptors, normal ducts have tended to express less mRNA than DCIS within the same section.

These data are expected to define the early molecular alterations in breast cancer, which may lead to therapeutic and preventative strategies.

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

PROJECT NUMBER

201 CB 09378-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Biological Consequences of Thrombospondin Expression

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

PI:	P. Steeg	Research Biologist	LP NCI
OTHER:	D. Weinstat-Saslow	Staff Fellow	LP NCI
	V. Zabrenetzky	Sr. Staff Fellow	LP NCI
	D. Roberts	Chief, Biochemical Pathology Section	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.6

PROFESSIONAL:

0.6

OTHER:

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

B

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

Thrombospondin (TSP) is an extracellular matrix component with multiple biological functions. Several reports have indicated an anti-angiogenic activity for TSP. In preliminary studies, we have noted decreased *thbs-1* mRNA expression in highly metastatic melanoma, lung and breast carcinoma cells, consistent with a suppressive activity.

In order to confirm the function of TSP, a *thbs-1* expression construct was transfected into the metastatic MDA-MB-435 breast carcinoma cell line. Injection of *thbs-1* and control transfected lines into nude mice indicated that TSP expression results in a significant decrease in primary mammary tumor size as well as incidence of distant metastatic disease. *In vitro* analysis to date indicate no difference in the anchorage-dependent or -independent growth of control and *thbs-1* transfected cells. Preliminary data using Factor VIII immunostaining, however, indicate a significant reduction in capillary density in primary tumors produced by *thbs-1* transfectants, indicative of an angiogenesis-suppressive effect. Should additional studies confirm an anti-angiogenic effect of TSP, characterization of the functional domains of the TSP protein will be conducted and tested in mice.

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

PROJECT NUMBER

Z01 CB 09131-09 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Role of Laminin Binding Proteins in Human Cancer

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

PI:	M. Sobel	Chief, Molecular Pathology Section	LP NCI
OTHER:	L. Bernstein	IRTA Fellow	LP NCI
	L. Wrathall	Biologist	LP NCI
	T. Simmons	Biologist	LP NCI
	L. Phuoc	Stay-in-School	LP NCI

COOPERATING UNITS (if any)

V. Castronovo, U. Liege, Belgium; T. Tennenbaum, LCCTP, DCE, NCI; F.-T. Liu, Scripps Clinic, La Jolla, CA; G. Taraboletti, Mario Negri Inst., Bergamo, Italy

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Laboratory of Pathology

SECTION

Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.6

PROFESSIONAL:

0.5

OTHER:

1.1

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

B

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

The interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. We have identified, isolated, characterized, and cloned three laminin binding proteins that are present in both normal and neoplastic tissues. All three proteins share a common epitope, and specifically bind to the poly-N-acetyllactosamine chains of laminin. The 67 kDa high affinity laminin receptor (67LR) has been previously characterized as a nonintegrin binding protein and has been molecularly cloned. It is expressed to a greater degree in metastatic tissues than in benign conditions in a variety of tissue-specific neoplasms. The 67LR is synthesized from a cytoplasmic precursor with an approximate molecular mass of 37 kDa. Using synthetic peptides, we have identified a 20 amino acid region of the precursor, designated peptide G, that binds directly to laminin with high affinity and that can inhibit attachment of laminin-coated melanoma cells to endothelium. We recently showed that this peptide enhances the metastatic potential of cancer cells via hematogenous routes. We have also identified a specific site on the laminin molecule to which the 67LR binds. We recently purified two other nonintegrin laminin binding proteins, HLBP31 and HLBP14, from human cancer cell lines. HLBP31 and HLBP14 have apparent molecular masses of 31 kDa and 14 kDa, respectively. We have used cDNA clones of the 67LR and the HLBP31 to assess the relative expression of mRNA in human colorectal carcinomas. The level of HLBP31 mRNA is inversely modulated with the 67LR in human colorectal carcinomas. Using the *in situ* hybridization technique, we established that the colonic cancer cells express more 67LR mRNA than do benign epithelial mucosal cells. Future studies will determine if the selective use of different laminin binding proteins by colonic cancer cells may play a functional role in the disease process.



Major Findings:

Identification of a potential laminin binding site on the 67LR. Previously, we identified a 20 amino acid domain of the 37 kD laminin receptor precursor ("peptide G") that had the ability to bind to laminin in *in vitro* experiments. Synthetic peptide G inhibited the attachment of laminin-coated melanoma cells to endothelium, thus inhibiting a critical step in the metastatic cascade. We therefore tested the ability of peptide G to inhibit metastatic spread of melanoma cells using an experimental metastasis model. The *in vivo* effect of peptide G and control peptides on melanoma cell lung retention time, on colonizing potential, on cell adhesion and chemotaxis to laminin, and on laminin binding was studied. *In vivo*, pretreatment of cells with peptide G resulted in an initial reduction in lung retention, as expected. However, unexpectedly, after 48 hours, there was a significant increase in both lung retention time and colonizing potential. *In vitro*, peptide G significantly increased laminin binding and cancer cell adhesion to laminin and subendothelial matrix, whereas chemotaxis to laminin was inhibited. We conclude that peptide G differentially affects the biological response of cancer cells to laminin. *In vitro*, it increases laminin binding and cell adhesion to subendothelial matrix. This might explain the *in vivo* effects of peptide G in augmenting long-term lung retention time and metastatic potential. The data suggest that direct adhesion of tumor cells to subendothelial matrix is a major pathway for hematogenous metastases of melanoma cells. Furthermore, tumor cell-matrix interactions (via laminin receptor binding to laminin) may be more relevant than tumor cell-endothelial cell attachment during blood-borne metastasis.

Biochemistry of the 67LR. Previous experiments suggested that the 67LR is synthesized from a cytoplasmic precursor with an apparent molecular mass of 37 kDa (37LRP). To further test this hypothesis, we have developed a series of antibodies directed against peptide domains of the 37 LRP. They have been affinity purified and tested for their ability to specifically recognize the 37 LRP and 67LR on immunoblots of cancer cell extracts as well as to immunoprecipitate both polypeptides from metabolically labeled cancer cell lysates. We have recently specifically eluted 37 kDa and 67 kDa polypeptides from immunoprecipitated cell products by using specific peptide antigens, and are currently purifying sufficient amounts of material for peptide mapping to provide biochemical evidence for the precursor-product relationship between the 37LRP and the 67LR.

Identification and cloning of HLBP14. We recently identified a 14 kDa polypeptide from human melanoma cells with laminin binding properties. This protein, designated HLBP14, was purified by laminin affinity chromatography and gel electroelution, digested with trypsin and cyanogen bromide, and the generated peptides were microsequenced. HLBP14 was homologous to the soluble L14,  $\beta$ -galactoside binding lectin. We have demonstrated that HLBP14 binds specifically to the poly-N-acetyllactosamine residues of human laminin and does not bind to other glycoproteins. Using reverse transcriptase-polymerase chain reaction technology, we have recently identified a clone of HLBP14 to study its expression in human cancers at the mRNA level.

Inverse modulation of expression of the 67LR and HLBP31. We recently identified a 31 kDa polypeptide from human melanoma cells with laminin binding properties. This protein, designated HLBP31, was purified by laminin affinity chromatography and gel electroelution. Cyanogen bromide and trypsin-generated peptides were

microsequenced. HLBP31 is homologous to the 31 kDa  $\beta$ -galactoside binding lectin, the IgE binding protein, and Mac-2 antigen. We have isolated a cDNA clone for HLBP31 and have obtained specific antibodies to HLBP31 from Dr. F.-T. Liu, Scripps Clinic. We have studied the expression of HLBP31 protein and mRNA using immunoblot and Northern blot analysis of human cancers of the colon. In contrast to increased levels of 67LR mRNA and protein in colon adenocarcinomas, HLBP31 mRNA and protein were decreased in the cancers compared to normal colonic mucosa. Expression of HLBP31 is also decreased in human skin cancers. Future studies will determine if the selective use of different laminin binding proteins plays a functional role in the disease process.

In situ hybridization using specific riboprobes of the 67LR. We have used the *in situ* hybridization technique to study the expression of 67LR mRNA in colorectal carcinoma cells compared to normal colonic cells and adenomas. We found that up-regulation of 67LR mRNA expression occurs relatively late in the progression of colonic carcinomas, since expression in adenomas was similar to that in normal mucosal cells.

#### Publications:

Campo E, Monteagudo C, Castronovo V, Claysmith AP, Fernandez PL, Sobel ME. Detection of laminin receptor mRNA in human cancer cell lines and colorectal tissues by *in situ* hybridization. *Am J Pathol* 1992;141:1073-83.

Cioce V, Margulies IMK, Sobel ME, Castronovo V. Interaction between the 67 kilodalton metastasis-associated laminin receptor and laminin. *Kidney Int.* 1993;43:30-7.

Castronovo V, Luyten F, van den Brule F, Sobel ME. Identification of a 14-kDa laminin binding protein (HLBP14) in human melanoma cells that is identical to the 14-kDa galactoside binding lectin. *Arch Biochem Biophys* 1992;297:132-8.

Castronovo V, Stetler-Stevenson WG, Sobel ME, Liotta LA. Molecular inhibition of cancer cell invasion and metastasis. In: Harris CC, Hirhashi S, Ito N, Pitot HC, Sugimura T, Terada M, Yokata J, eds. *Multistep carcinogenesis. Proceedings of the 22nd International Symposium of the Princess Takamatsu Cancer Research Fund, Tokyo, 1991.* Boca Raton: CRC Press, 1992;319-37.

Castronovo V, Campo E, van den Brule F, Claysmith AP, Cioce V, Liu F-T, Fernandez PL, Sobel ME. Inverse modulation of steady state mRNA levels of two non-integrin laminin binding proteins in human colon carcinoma. *J Natl Cancer Inst* 1992;84:1161-9.

Taraboletti G, Belotti D, Giavazzi R, Sobel ME, Castronovo V. Enhancement of metastatic potential of murine and human melanoma cells by laminin receptor peptide G: attachment of cancer cells to the subendothelial matrix as a pathway for hematogenous metastases. *J Natl Cancer Inst* 1993;85:235-40.

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

PROJECT NUMBER

Z01 CB 09353-03 I.P.

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

**Differential Gene Expression in Gynecological Tumors**

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

PI:	M. Sobel	Chief, Molecular Pathology Section	LP NCI
OTHER:	S. Lesenfants	Visiting Scientist	LP NCI
	A. Chariot	General Fellow	LP NCI
	A. Alag	Special Volunteer	LP NCI
	L. Phuoc	Stay-in-School	LP NCI

COOPERATING UNITS (if any)

Dr. Y.E. Shi; Georgetown Univ. Med. School, Washington, D.C.; Dr. A. Berchuck, Duke University, North Carolina

LAB/BRANCH

Laboratory of Pathology

SECTION

Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.7

PROFESSIONAL:

1.4

OTHER:

1.3

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 initiated a series of survey studies of breast and ovarian cancers to determine if specific genes are differentially expressed in those gynecological tumors that go on to metastasize. We are looking specifically at the expression of cell surface receptors such as the 67 kDa high affinity laminin receptor (67LR) and HLBP31 (a 31 kDa laminin binding protein), at genes involved in cell proliferation such as ribonucleotide reductase, as well as genes involved in cell development and differentiation such as homeobox genes. To accomplish this, we have recently cloned the catalytic subunit of human ribonucleotide reductase, HLBP31, and several human homeobox genes from breast cancer cells. Freshly frozen and fixed tumor specimens with matched normal tissue controls are being analyzed at both the protein and RNA levels using specific antibodies and cDNA probes. Western immunoblot, immunohistochemistry, Northern blot, and *in situ* hybridization techniques are being used to assess specific expression. Results will be correlated with clinical parameters and patient survival to establish the prognostic values of the systematic detection of these genes in gynecological tumors. As an adjunct to these survey studies, *in vitro* experiments were conducted to determine the specific effect of steroid hormones on human breast cancer cells. In breast cancers, 67 LR and HLBP31 are inversely modulated. The 67LR is upregulated by progesterone in human breast cancer cells. Several polymorphisms/mutations have been detected in homeobox genes in the human breast carcinoma-derived cell line MCF7. The role of these alterations is being examined in breast cancers.

Major Findings:

Effect of progestins on expression of the 67 kD laminin receptor in human breast cancer cells. We have previously shown that the expression of the 67 kD laminin receptor (67LR) is increased in steroid receptor-negative human breast cancer cell lines compared to steroid-responsive cell lines such as T47D. We have now studied the expression of the 67LR in subclones of T47D with different invasive potential. The expression of 67LR protein (as detected on immunoblots) and 67LR mRNA (on Northern blots) was increased in the more invasive cell line T47Dco compared to the poorly invasive parental line. Furthermore, treatment of T47D cells with synthetic progestin R5020 resulted in an increase in 67LR expression and in increased attachment to laminin. A new antiprogestin, ZK 112.993, inhibited progestin-stimulated 67LR expression and the increased attachment to laminin. These results suggest that progestins may play a role in mediating the invasive properties of steroid receptor positive human breast cancer cells via the laminin-67LR pathway.

Inverse modulation of 67LR and HLBP31 in human breast cancers. We have studied the expression of two non-integrin laminin binding proteins in human breast cancers. The 67LR is a 67 kD laminin receptor whose expression is increased in metastatic breast cancers. HLBP31 is another laminin binding protein. We previously demonstrated that expression of the HLBP31 protein and mRNA is inversely modulated compared to 67LR expression in human colon cancers. Immunohistochemical staining of fixed human breast cancer specimens, using specific antibodies to 67LR and HLBP31, shows that HLBP31 expression is decreased in invasive human breast cancers. As expected, 67LR expression is increased in the metastatic specimens.

Increased expression of the 67LR in human ovarian cancers. The expression of the 67LR in 30 ovarian cancer specimens at the mRNA and protein levels was studied using Northern blot, immunoblot, and immunohistochemical staining techniques. Expression of the 67LR was consistently increased in the cancer specimens, and was significantly increased in the group of patients whose cytoreductive surgery was suboptimal. Suboptimal debulking at the time of surgical diagnosis is correlated with higher morbidity and mortality. Compared to other parameters, such as histological tumor grading and surgical staging, 67LR expression and suboptimal debulking were the best predictors of poor outcome. In comparison, expression of another laminin binding protein, HLBP31, did not significantly differ. These studies suggest that expression of the 67LR is altered in ovarian carcinomas and that the 67LR may play a role in the invasive and metastatic spread of ovarian cancers.

Altered expression of ribonucleotide reductase in ovarian cancers. Ribonucleotide reductase catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, a rate limiting step of DNA synthesis. Expression of the catalytic (M2) subunit of the enzyme is cell cycle (S-phase) dependent. We have hypothesized that cancer cells that proliferate rapidly may have an overall increase in the expression of the M2 subunit. We have molecularly cloned the human M2 cDNA and have used it as a probe to determine mRNA levels in human specimens. In preliminary studies of expression of M2 mRNA

in human ovarian cancer specimens, patients with poor clinical outcome had higher levels of mRNA than did patients with longer survival. These studies are being continued to determine how well M2 mRNA levels correlate with more traditional indices of proliferation such as the mitotic index and expression of Ki67 antigen.

Expression of homeobox genes in human breast cancer cells. Homeobox genes, encoding transcriptional regulators, act in complex regulatory cascades to control the coordinated expression of genes involved in specific developmental processes. We have asked the question whether specific homeobox genes may control the coordinated expression of genes involved in human cellular transformation and in tumor invasion and metastasis of human breast cancers. We constructed a cDNA library from the human breast cancer-derived cell line MCF7 and exhaustively screened it using a highly conserved 183 base pair DNA segment of all known homeobox genes. We identified and purified 37 clones. DNA restriction enzyme analysis and sequencing identified these as representative of 6 different homeobox genes: A1, A4, A10, B5, B7, and C6. We identified a unique alternate splicing event in HoxC6, in which previously identified intron sequences were present in the expressed mRNA from MCF7 as well as other human cell lines. In addition, a base change was identified in 6 different Hox C6 cDNA isolates, resulting in an amino acid change from methionine to leucine in the predicted homeobox protein sequence. In the untranslated 3' region of the HoxC6 cDNA clones, multiple base changes were also noted. Polymorphisms were also identified in some of the Hox B7 cDNAs, resulting in a potential change in the length of the homeobox protein. In the 3' untranslated region of Hox A4, a 50 base pair insertion was identified. The physiologic significance of these polymorphisms/mutations is under investigation.

#### Publications:

van den Brule F, Engel J, Stetler-Stevenson WG, Liu-F-T, Sobel ME, Castronovo V. Genes involved in tumor invasion and metastasis are differentially modulated by estradiol and progestin in human breast cancer cells. *Int J Cancer* 1992;52:653-7.

Shi YE, Turri J, Yieh L, Sobel ME, Yamada Y, Lippmann ME, Dickson RB, Thompson EW. Expression of 67 kDa laminin receptor in human breast cancer cells: regulation by progestins. *Clin Exp Metast* 1993;11:251-61.

Minafra S, Luparello C, Pucci-Minafra I, Sobel ME, Garbisa S. Adhesion of 8701-BC breast cancer cells to type V collagen and 67 kDa receptor. *J Cell Sci* 1992;102:323-8.

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

PROJECT NUMBER

Z01 CB 09368-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Molecular Pathology Resource Center

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

PI:	M. Sobel	Chief, Molecular Pathology Section	LP NCI
OTHER:	L. Wrathall	Biologist	LP NCI
	T. Simmons	Biologist	LP NCI
	A. Alag	Special Volunteer	LP NCI
	G. Senterre	Special Volunteer	LP NCI
	A. Chariot	General Fellow	LP NCI
	L. Phuoc	Stay-in-School	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.6

PROFESSIONAL:

0.3

OTHER:

2.3

CHECK APPROPRIATE BOXES:

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

D

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

A molecular pathology resource center is being developed to coordinate the training of residents, intramural staff, and extramural scientists on the application of molecular pathology techniques to the study of cancer biology. The center will provide a resource with tissue specimens for the analysis of gene expression in neoplasia. In addition, bench and didactic training in molecular techniques will be provided. We have provided training to three postgraduate students.

Major Accomplishments:

During the planning stages of the resource center, we have begun to provide training to extramural and intramural scientists. Training includes bench experience with techniques including Northern blot, Southern blot, immunoblot, RNA and DNA extraction, in situ hybridization, probe labeling, and tissue handling. In addition, participants participate in a weekly molecular pathology journal club as well as weekly data review sessions. Didactic lectures on selected topics are also provided several times a week. We have provided training to a pharmacist, a graduate student from Boston University on an Oncobiology Training Grant, and a foreign medical student. Training is provided for short (three months) as well as long time periods (one year) depending on the needs of the participant. In addition, equipment for retrieval and storage of tissues for analysis are being acquired for the future expansion of this program.

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

PROJECT NUMBER

Z01 CB 09382-01 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Tumor Promotion in Mouse Epidermal Cells

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

PI:	L. Bernstein	IRTA Fellow	LP NCI
OTHER:	M. Sobel	Chief, Molecular Pathology Section	LP NCI

COOPERATING UNITS (if any)

Dr. N. Colburn, LVC, FCRDC

LAB/BRANCH

Laboratory of Pathology

SECTION

Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.6

PROFESSIONAL:

0.5

OTHER:

0.1

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 JB6 cell system of mouse epidermal cells consists of a battery of clonal genetic variants in various stages of progression toward a neoplastic endpoint. These cells provide a model system for evaluating rate limiting and essential components in the signal transduction apparatus for neoplastic transformation induced by tumor promoting agents. Previously, we reported that cells which are sensitive to promotion of transformation by tumor promoters (called P+ cells) have tumor promoter inducible AP-1 transcriptional activity, whereas cells resistant to promotion of transformation by tumor promoters (called P- cells) do not. c-Jun protein expression and P-1 activity are required for P+ phenotypic responsiveness to tumor promoters since dominant negative c-Jun mutants block c-Jun expression, AP-1 activity and tumor promoter induced neoplastic transformation. Basal and induced levels of c-Jun mRNA and protein are elevated in P+ cells compared to P- cells, whereas levels of other Jun family members are equal in P+ and P- cells, as are levels of c-Fos, Fra-1 and Fos B. However, levels of a novel protein species immunoprecipitated by anti-Fra-1 antibodies, and which we designate as "Fra-1 related protein" or Fra-1 RP, are induced by tumor promoting agents in P- cells but not P+ cells. c-Jun and Fra-1 form a complex in JB6 cells to which Fra-1 RP may be bound via c-Jun. Currently, we are engaged in detailed characterization of Fra-1 RP and its association with the AP-1 transcription complex.



Major Findings:

Fra-1 related protein (Fra-1 RP) is an apparently novel protein species which is immunoprecipitated by antibodies against Fra-1 protein. Six independent antisera which recognize Fra-1 immunoprecipitate Fra-1 RP. Levels of Fra-1 RP are induced by the tumor promoting agents tetradecanoyl phorbol-13-acetate (TPA) and epidermal growth factor (EGF) in promotion resistant JB6 cells but not in promotion sensitive JB6 cells. Induction of Fra-1 RP levels is maximal after 90 minutes of tumor promoter treatment. Antisera against c-Jun immunoprecipitate an antigen with the molecular weight of Fra-1 RP. Two dimensional electrophoretic analyses of anti-Fra-1 immunoprecipitates demonstrate co-precipitation of c-Jun, and Fra-1. Therefore, c-Jun and Fra-1 form a complex in JB6 cells. We hypothesize that the association of Fra-1 RP with Fra-1 is indirect via its association with c-Jun. Future analyses are aimed at characterization and cloning of Fra-1 RP. Since it is induced by tumor promoting agents specifically in P- cells, it may behave as a suppressor of promotion of transformation.

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

PROJECT NUMBER

Z01 CB 00550-13 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Jaffe	Chief, Hematopathology Section	LP NCI
OTHER:	L. Medeiros	Senior Staff Fellow	LP NCI
	D. Longo	Senior Investigator	BRMP NCI
	M. Raffeld	Senior Staff Fellow	LP NCI
	M. Stetler-Stevenson	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 STAFF 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 unrounded 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.

Selected cases of hematologic malignancies are also referred for detailed immunophenotypic, genotypic, and morphologic analysis. Such cases are selected for unusual clinical and/or histologic features.

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:

Common variable immunodeficiency syndrome is a disease reported to have an increased incidence of non-Hodgkin's lymphoma. The relative risk has been reported to range from 30-fold to 438-fold. For most of the reported non-Hodgkin's lymphomas, immunophenotypic and molecular analyses have not been performed. We reviewed our experience with 30 nodal and extranodal lymphoid lesions from 17 patients with common variable immunodeficiency syndrome. Immunohistochemical and molecular studies were performed. The biopsies were classified into 4 groups: malignant lymphoma (2 cases), atypical lymphoid hyperplasia (8 cases), reactive lymphoid hyperplasia (14 cases) and chronic granulomatous inflammation (6 cases). The cases of atypical lymphoid hyperplasia were of particular interest, as these patients had either widespread involvement or massive disease. The diagnosis of lymphoma was considered likely by the clinicians, and in three cases the histologic slides were originally interpreted as malignant lymphoma by the referring pathologist. Although the architecture of these lesions appeared to be effaced, immunohistochemical and molecular analysis failed to support a malignant diagnosis. In addition, clinical follow-up of these patients was benign. We conclude that the majority of the lymphoid lesions in patients with CVID are benign, and that the risk of lymphoma may have been overestimated in the past. Two immunoblastic lymphomas were identified and EBV was demonstrated in one by *in situ* hybridization.

A series of small noncleaved B-cell lymphomas associated with a florid epithelioid granulomatous response was published. The epithelioid granulomatous response masked the malignant proliferation and led to a delay of diagnosis in one case. In addition, the florid granulomatous reaction appeared to be associated with an excellent prognosis. Extended clinical follow-up was available in five patients, all of whom achieved a complete remission and are alive without evidence of disease (median follow-up 6 years).

Publications:

Medeiros LJ, Mandava SK, Naylor P, Fowler D, Jaffe ES, Stetler-Stevenson M. Malignant thymoma associated with T-cell lymphocytosis: A case report with immunophenotypic and gene rearrangement analysis. Arch Pathol Lab Med 1993;117:279-83.

Zarate-Osorno A, Medeiros LJ, Jaffe ES. Hodgkin's disease coexistent with plasma cell dyscrasia. Arch Pathol Lab Med 1992;116:969-72.

Banks PM, Chan J, Cleary ML, Delsol G, De Wolf-Peters C, Gatter K, Grogan TM, Harris NL, Isaacson PG, Jaffe ES, Mason D, Pileri S, Ralfkiaer E, Stein H, Warnke RA. Mantle cell lymphoma: A proposal for unification of morphologic, immunologic and molecular data. Am J Surg Pathol 1992;16:637-40.

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- Sneller MC, Straus SE, Jaffe ES, Fleisher TA, Stetler-Stevenson M, Strober W. A novel lymphoproliferative/autoimmune syndrome resembling murine lpr/gld disease. *J Clin Invest* 1992;90:334-41.
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- Zarate-Osorno A, Medeiros LJ, Longo DL, Jaffe ES. Non-Hodgkin's lymphomas arising in patients successfully treated for Hodgkin's disease: A clinical, histologic, and immunophenotypic study of 14 cases. *Am J Surg Pathol* 1992;16:885-95.
- Sander CA, Medeiros LJ, Weiss LM, Yano T, Sneller MC, Jaffe ES. Lymphoproliferative lesions in patients with common variable immunodeficiency syndrome. *Am J Surg Pathol* 1992;16:1170-82.
- Thomas RM, Jaffe ES, Zarate-Osorno A, Medeiros LJ. Inflammatory pseudotumor of the spleen: A clinicopathologic and immunophenotypic study of eight cases. *Arch Pathol Lab Med* (in press)
- Zarate-Ossorno A, Jaffe ES, Medeiros LJ. Metastatic nasopharyngeal carcinoma initially presenting as cervical lymphadenopathy: A report of two cases that resembled Hodgkin's disease. *Arch Pathol Lab Med* 1992;116:862-5.
- Monterrose V, Jaffe ES, Merino MJ, Medeiros LJ. Malignant lymphomas involving the ovary - A clinicopathologic analysis of 39 cases. *Am J Surg Pathol* 1993;17:154-70.
- Zarate-Osorno A, Medeiros LJ, Kingma DW, Longo DL, Jaffe ES. Hodgkin's disease following non-Hodgkin's lymphoma - A clinicopathologic and immunophenotypic study of nine cases. *Am J Surg Pathol* 1993;17:123-32.

Parveen T, Navarro-Román L, Raffeld M, Jaffe ES. Low-grade B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) arising in the kidney. Arch Pathol Lab Med (in press)

Hollingsworth HC, Longo DL, Jaffe ES. Small noncleaved cell lymphoma associated with florid epithelioid granulomatous response--A clinicopathologic study of seven patients. Am J Surg Pathol 1993;17:51-9.

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

PROJECT NUMBER

Z01 CB 00855-11 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Pathologic Features of Viral Associated Lymphoproliferative Disorders

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

PI:	E. Jaffe	Chief, Hematopathology Section	LP NCI
OTHER:	W. Blattner	Senior Investigator	EEB NCI
	P. Levine	Senior Investigator	EEB NCI
	M. Raffeld	Senior Investigator	LP NCI
	M. Stetler-Stevenson	Senior Staff Fellow	LP NCI
	L. Medeiros	Senior Staff Fellow	LP NCI
	L. Roman	Medical Staff Fellow	LP NCI
	D. Kingma	Clinical Associate	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF 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.

Other diseases are being investigated with respect to a possible viral association: angiocentric immunoproliferative disorders (lymphomatoid granulomatosis), sinus histiocytosis with massive lymphadenopathy, systemic Castleman's and Kikuchi's disease, and non-Hodgkin's lymphomas. Viruses under investigation include EBV, HHV-6, HTLV-I, and HTLV-II.

Major Findings:

We continued our studies regarding the role of viruses in lymphoproliferative disorders. A study was published which identified HHV-6 by *in situ* hybridization in the abnormal histiocytes of sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease).

Our studies of the role of Epstein-Barr virus (EBV) in angiocentric T-cell lymphomas have been extended. There is epidemiologic evidence that angiocentric lymphomas vary in their incidence, and that the incidence of this disease is associated with the prevalence of EBV infections. For example, angiocentric lymphomas are common in Asian countries where EBV associated malignancies are also seen at increased incidence. We identified a cluster of nasal lymphomas in Peru. Clinically and pathologically the disease is similar to that seen in China and Japan, and is associated with destructive midline nasal lesions. An immunophenotypic and viral analysis of these cases was completed. It showed a T-cell associated phenotype, consistent with angiocentric lymphoma, and a very high prevalence of Epstein-Barr virus. EBV was identified by *in situ* hybridization in all cases expressing a T-cell phenotype.

Studies of EBV associated lymphoproliferative disease were extended to include malignant lymphomas occurring in immunodeficiency states. A study of the lymphoproliferative lesions in patients with common variable immunodeficiency syndrome was completed. Although most of the lymphoid lesions were felt to be atypical but reactive, two instances of lymphoma were identified. In one such case studied by *in situ* hybridization, EBV was identified in the neoplastic cells. Therefore, EBV appears to play an important pathogenetic role in the malignant lymphomas arising in common variable immunodeficiency syndrome, as well as other immunodeficiency states.

A rare case of T-cell lymphoma associated with Epstein-Barr virus in a patient receiving immunosuppression for a renal transplant was also identified. This appears to be the first reported incidence of a transplant associated T-cell lymphoma associated with the Epstein-Barr virus.

Our studies of Hodgkin's disease occurring in association with chronic lymphocytic leukemia were extended to study the pathogenesis of this process. Mediation by Epstein-Barr virus appears to play an important role, since EBV was identified by *in situ* hybridization in the Reed-Sternberg cells and mononuclear cells of all cases of Hodgkin's disease, but not in the associated chronic lymphocytic leukemia lymphocytes.

Finally, our studies of the epidemiology of HTLV-1 associated lymphoma were extended to Singapore where a low incidence of this disease process was identified. The incidence in Singapore is less than that seen in other endemic regions in the Pacific basin.

Publications:

- Medeiros LJ, Mandava SK, Naylor P, Fowler D, Jaffe ES, Stetler-Stevenson M. Malignant thymoma associated with T-cell lymphocytosis: A case report with immunophenotypic and gene rearrangement analysis. *Arch Pathol Lab Med* 1993;117:279-83.
- Zarate-Osorno A, Medeiros LJ, Jaffe ES. Hodgkin's disease coexistent with plasma cell dyscrasia. *Arch Pathol Lab Med* 1992;116:969-72.
- Banks PM, Chan J, Cleary ML, Delsol G, De Wolf-Peters C, Gatter K, Grogan TM, Harris NL, Isaacson PG, Jaffe ES, Mason D, Pileri S, Ralfkiaer E, Stein H, Warnke RA. Mantle cell lymphoma: A proposal for unification of morphologic, immunologic and molecular data. *Am J Surg Pathol* 1992;16:637-40.
- Levine PH, Jahan N, Murari P, Manak M, Jaffe ES. Detection of human herpesvirus-6 in tissues involved by sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease). *J Infect Dis* 1992;166:291-5.
- Jaffe ES, Zarate-Osorno A, Medeiros LJ. The interrelationship of Hodgkin's disease and non-Hodgkin's lymphomas--lessons learned from composite and sequential malignancies. *Semin Diag Pathol* 1992;9:297-303.
- Jaffe ES, Raffeld M, Medeiros LJ, Stetler-Stevenson M. An overview of the classification of non-Hodgkin's lymphomas: An integration of morphological and phenotypical concepts. *Cancer Res (suppl)* 1992;52:5447s-5452s.
- Momose H, Jaffe ES, Shin SS, Chen Y-Y, Weiss LM. Chronic lymphocytic leukemia/small lymphocytic lymphoma with Reed-Sternberg-like cells and possible transformation to Hodgkin's disease: Mediation by Epstein-Barr virus. *Am J Surg Pathol* 1992;16:859-67.
- Arber DA, Weiss LM, Albújar PF, Chen Y-Y, Jaffe ES. Nasal lymphomas in Peru: High incidence of T-cell immunophenotype and Epstein-Barr virus infection. *Am J Surg Pathol* 1993;17:392-9.
- Navarro-Román L, Román GC, Katz D, Jaffe ES. Human T-lymphotropic virus type I (HTLV-I). In: Schwartz DA, Connor DH, Chandler FW, eds. *Diagnostic pathology of infectious disease: A text and atlas*. Appleton & Lange, Publ. (in press)
- Sng I, Levin A, Jaffe ES, Ng HW, Sim CS, Blattner WB. T-cell lymphoma in Singapore: pathology, clinical findings and association with HTLV-1 antibodies. *Histopathology* 1992;21:101-13.
- Abruzzo LV, Schmidt K, Weiss LM, Jaffe ES, Medeiros LJ, Sander CA, Raffeld M. B-cell lymphoma following angioimmunoblastic lymphadenopathy: A case with oligoclonal gene rearrangements associated with Epstein-Barr virus. *Blood* (in press)
- Kumar S, Kumar D, Kingma DW, Jaffe ES. Epstein-Barr virus associated T-cell lymphoma in a renal transplant patient. *Am J Surg Pathol* (in press)



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

PROJECT NUMBER

Z01 CB 09182-05 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Molecular Biology of Lymphoproliferative Diseases: Applied Studies

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

PI:	M. Raffeld	Medical Officer (Path)	LP NCI
OTHER:	T. Yano	Visiting Associate	LP NCI
	C. Sander	Guest Researcher	LP NCI
	H. Clark	Visiting Fellow	LP NCI
	E. Jaffe	Chief, Hematopathology Section	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.3

PROFESSIONAL:

1.3

OTHER:

CHECK APPROPRIATE BOXES)

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

A, B

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

The overall goal of this project is to generate molecular profiles of the lymphoproliferative disorders and to assess the usefulness of these profiles in providing clinically relevant information that may have both diagnostic and prognostic value.

Molecular profiles are generated using genetic loci that have been associated with particular lymphoproliferative disorders such as bcl-1, bcl-2 and c-myc as well as other loci whose influence in these disorders are not so well understood. The data generated for each locus is correlated with clinical, immunologic, and pathologic data. Using this combined approach, we hope to increase our diagnostic and prognostic precision in the classification of lymphopoietic disorders.

The molecular profiles also generate important biologic information with regard to the particular genes under study. Structural analysis of an abnormal gene or gene product allows us to acquire information concerning the functioning of that gene in the lymphoma and its effect on the biologic behavior of the lymphoma cell.

Sensitive molecular techniques are being developed in order to improve our ability to diagnose minimal disease, monitor the effect of therapy, and predict recurrences. Techniques designed to make better use of routine pathologic materials, particularly fixed paraffin embedded tissues, are being developed. Non-radioactive approaches are being explored so that molecular diagnostics may be performed in routine clinical laboratories as well as in academic centers.

Major Findings:

We previously reported that the bcl-1 major breakpoint region is associated with mantle cell lymphoma. We have now completed a larger study that examines several additional minor breakpoint regions and we have also studied the expression of the bcl-1 related gene bcl-1/PRAD1. These studies have shown that bcl-1 rearrangement and bcl-1/PRAD1 expression are specific to the mantle cell lymphomas.

Previously we performed a molecular study of small non-cleaved lymphomas and showed that molecular differences exist between the Burkitt's subgroup and the non-Burkitt's subgroup. In the course of these studies, we identified an aggressive subgroup of lymphomas associated with particular abnormalities of the MYC gene. We also discovered that the MYC gene is commonly mutated in its second exon in Burkitt's lymphoma. Furthermore, we found that these mutations were clustered within particular regions and codons. We are following up these observations with functional studies using the mutated MYC proteins.

We have determined the frequency with which each of the breakpoint regions of bcl-2 are involved in translocation and are continuing to investigate whether specific breakpoints might influence the clinical behavior of lymphoma. Follicular lymphomas that are negative for bcl-2 rearrangement are being studied for expression of bcl-2 as part of an effort to understand the differences between the bcl-2 rearranged and non-rearranged cases.

To adapt PCR technology to the diagnosis of t(14;18) translocated lymphomas, we have developed sets of oligonucleotide primers specific for each of four reported breakpoint clusters so that the majority of t(14;18) translocated lymphomas can be identified. We are continuing to study the feasibility of using PCR to follow response to therapy and predict relapse using peripheral blood samples and other tissue samples.

We have developed sets of primers specific to the immunoglobulin gene that are capable of identifying up to 75% of all heavy chain VDJ rearrangements and are using these primers to complement the bcl-2/JH primer pairs and to extend our PCR analysis to non t(14;18) translocated lymphomas.

We have also extended our ability to study clonal populations of T cells by PCR by using primers to the T-cell receptor  $\gamma$  chain gene and to the T-cell receptor  $\beta$  chain gene.

Publications:

Raffeld M, Sander CA, Yano T, Jaffe ES. Mantle cell lymphoma: an update. *Lymphoma & Leukemia* 1992;8:161-66.

Yano T, van Krieken JHJM, Magrath IT, Longo DL, Jaffe ES, Raffeld M. Histogenetic correlations between subcategories of small non-cleaved cell lymphomas. *Blood* 1992;79:1282-90.

Yano T, Sander CA, Clark HM, Dolezal MV, Jaffe ES, Raffeld M. Clustered mutations in the second exon of the MYC gene in sporadic Burkitt's lymphoma. *Oncogene* (in press)

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

PROJECT NUMBER

Z01 CB 09191-04 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Molecular Biology of Disease Progression in Lymphoproliferative Diseases

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

PI:	M. Raffeld	Medical Officer (Path)	LP NCI
OTHER:	T. Yano	Visiting Associate	LP NCI
	H. Clark	Visiting Fellow	LP NCI
	C. Sander	Guest Researcher	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.6

PROFESSIONAL:

1.6

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A, B

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

The overall goal of this project is to define the molecular events involved in the transformation of low grade lymphomas to more aggressive forms.

We have chosen follicular lymphoma as our primary model because it is a homogeneous group characterized by a single molecular lesion (bcl-2 translocation and deregulation), because over 70% of these low grade lymphomas will evolve into a histologically distinct high grade lymphoma, and because it is the most common low grade lymphoma in the United States.

We have accumulated a large series of progressed follicular lymphomas from patients that have had multiple biopsies over the course of their disease. The matched biopsies from individual patients are studied for a variety of phenotypic and genotypic characteristics. A major focus is to identify acquired alterations within genes that have been previously implicated in growth and cell cycle control (e.g.: MYC, p53, Rb and PCNA). Changes in expression levels are also studied. A major focus is to identify molecular markers that will be predictive of poor prognosis or histologic progression so that appropriate preventive therapeutic decisions can be made early.

In order to identify additional genetic loci that may play a significant role in lymphoma progression, we are proceeding with the development of differential gene expression assays. Using this approach, we hope to identify other critical genes that may be important to the progressed phenotype.

Major Findings:

Previously we found that the bcl-2 gene itself was unaffected by progression and that other genes must be contributing to the dramatic changes in cellular morphology and biologic behavior that occur following transformation. In the past year, we have completed initial studies on the involvement of c-myc and have found acquired, progression related structural changes in this gene in approximately 10% of progressed lymphomas. The precise molecular change and its biologic significance is being pursued.

We have recently completed another study on the involvement of the bcl-3 (17q22) gene (another anecdotally reported progression related locus) and again have found abnormalities of this locus in 10-15% of the progressed lymphomas. In contrast to the situation for c-myc, these changes are not temporally associated with the progression event, and we have concluded that alterations of this gene do not directly result in progression but may predispose to progression.

We have completed a study of the role of the P53 gene in progression using SSCP and sequencing technology. We have found acquired mutations of this gene in approximately 1/3 of our progressed follicular lymphomas, while the antecedent low grade follicular lymphomas did not possess mutations. Interestingly, it is possible to find rare and/or clusters of p53 overexpressing cells in the indolent lymphoma phase suggesting that mutations that lead to overexpression occur early, but do not disseminate until later, at or near the time of progression.

Ras family genes are infrequently involved in progression.

Other genes and cofactors that do not appear to have a role in progression include the CLL progression related gene bcl-3 (19q13) and EBV.

Additional genes currently under investigation include c-rel, Rb, PCNA and mdm-2.

We are also in the process of performing parallel studies on a smaller number of progressed small lymphocytic lymphomas (Richter's syndrome).

Publications:

Yano T, Longo DL, Jaffe ES, Raffeld M. MYC rearrangements in histologically progressed follicular lymphomas. Blood 1992;80:756-67.

Yano T, Sander CA, Andrade RE, Gauwerky CE, Croce CM, Longo DL, Jaffe ES, Raffeld M. Molecular analysis of the BCL-3 locus at chromosome 17q22 in B-cell neoplasms. Blood (in press)

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

PROJECT NUMBER

Z01 CB 09366-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Polymerase Chain Reaction Systems for T/B Clonality in Lymphoid Neoplasms

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

PI:	T. Greiner	Clinical Associate	LP NCI
OTHER:	E. Jaffe	Chief, Hematopathology Section	LP NCI
	M. Raffeld	Medical Officer	LP NCI
	D. Kingma	Clinical Associate	LP NCI

COOPERATING UNITS (if any)

Fred Dick, M.D., University of Iowa, Department of Pathology  
 Randy Gascoyne, M.D., British Columbia Cancer Center

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

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

A

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

The overall goal of this project is to utilize the polymerase chain reaction (PCR) system to identify clonal gene rearrangements in lymphoid neoplasms. The purposes include the following: detect clonality to support diagnostic work of cases, investigate and characterize lymphoid diseases, and provide sensitive methods to follow patients.

Previous work in the section to identify clonal gene rearrangements has been primarily based on using Southern blot technology. However, the sensitivity is low, the methodology requires 1-2 weeks for completion, and can not be performed on DNA extracted from paraffin embedded tissue. Methods have been described using the polymerase chain reaction to amplify clonal rearrangements in both T and B cell lineages. The section has much experience performing PCR for bcl-2 in follicular lymphomas as a method to identify lymphoid clones. No PCR work has previously been done in the section with the T cell system prior to this report.

T cell gene rearrangements can now be amplified from both fresh tissue and paraffin embedded tissue, including tumors such as acute lymphocytic leukemia, peripheral T cell lymphoma, mycosis fungoides, gamma delta lymphomas, subcutaneous T cell lymphomas, and other lymphomas. A high resolution system using denaturing gradient gel electrophoresis has been modified to follow the unique gene rearrangement for each patient's tumor in serial surgical biopsies, cytology aspirations, or peripheral blood samples. B cell immunoglobulin gene rearrangements can be amplified and this is being used to study the lymphocyte predominant form of Hodgkin's disease. This work provides the basis to do PCR *in situ* on paraffin tissue sections to identify specific cell types involved.

Major Findings:

Dr. Greiner, during prior work at the University of Iowa, had developed PCR primers to amplify some T cell receptor gamma gene rearrangements in acute lymphocytic leukemia and T cell lymphoma. Gene rearrangements were sequenced, and patient specific primers were developed to detect minimal residual disease in follow-up bone marrow samples. In addition, PCR amplification methods to rapidly amplify paraffin embedded tissue were developed.

Primers have been designed and shown to amplify the whole spectrum of T cell receptor gamma rearrangements. Specific genes used in the rearrangements can be identified and there is excellent correlation with Southern blot results.

Denaturing gradient gel electrophoresis has been adapted for the analysis of amplified gene rearrangements to provide superior resolution of genes based on the tumor's unique DNA sequence. Correlation with the DNA sequence and T gamma genes used has been done with 10 cases of acute lymphocytic leukemia (Fred Dick, M.D., collaboration). Diseases that are currently being studied include: peripheral T cell lymphoma, mycosis fungoides, gamma delta lymphomas, subcutaneous T cell lymphomas. We have demonstrated additional evidence that granulomatous slick skin disease is a preneoplastic T-cell lymphoma skin condition. Plans are underway to study angioimmunoproliferative disease, angioimmunoblastic lymphadenopathy, lymphomatoid papulosis, T gamma lymphoproliferative disease, and other lymphoid lesions.

A second accomplishment has been the ability to amplify T cell gamma gene rearrangements from paraffin embedded tissues. This provides a significant tool to the clinical diagnostic and research study of lymphoid neoplasms which have no fresh tissue available for Southern blots. This has been especially applicable for the lymphomas that arise in the skin, or extranodal cases, where routinely pathologists fix entire specimens in formalin. The technique can be performed in two days, adding a rapid time improvement.

The relationship of B cell lymphomas to Hodgkin's disease is being addressed (in collaboration with Randy Gascoyne, M.D.) by using PCR amplification of B cell gene rearrangements. The pilot study has successfully amplified rearrangements in 9 cases. B cell lymphomas arising in AIDS patients and tumors arising in bone marrow transplant patients may also be studied that have only paraffin tissue available.

With the above accomplishments, work has been attempted to develop in situ PCR for both T and B cell neoplasms to investigate cell types in tumors, probe for residual disease, and study the nature of lymphoma-like diseases.

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

PROJECT NUMBER

201 CB 09372-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Aneuploidy and Cell Cycle Fractions in Benign and Malignant Tumors

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

PI:	M. Stetler-Stevenson	Medical Officer	LP NCI
OTHER:	S. Sebers	Medical Technologist	LP NCI
	J. McClanahan	Medical Technologist	LP NCI

COOPERATING UNITS (if any)

NIDDKD, Digestive Diseases Branch

LABORATORY

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

0.75

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

We have studied the presence of aneuploidy and S phase in specimens containing gastrinomas from patients with Zollinger-Ellison syndrome. All patients with multiple stem line aneuploidy had widespread disease while all patients with a single aneuploid population had localized disease. The correlation between disease and ploidy was significant with  $P=0.00022$ . Our findings also suggest that ploidy may be of prognostic importance in patient survival; however, a longer follow-up time and greater number of patients is necessary to determine if this is significant. High S-phase correlated significantly with widespread disease ( $P=0.0039$ ). Therefore, DNA content analysis of gastrinomas provides important information that can be utilized by the clinicians in treatment decisions. More aggressive therapy in patients with multiple stem line aneuploidy or high S-phase may prevent metastases and widespread disease.

We are studying the DNA content of thyroid carcinomas from children and adults in the region contaminated with radiation from the Chernobyl accident, adults in the United States with a history of radiation exposure, and American adults with no history of radiation exposure. We will study the correlation between a history of radiation exposure and the incidence of aneuploidy as well as S phase in thyroid carcinoma. The prognostic value of DNA content analysis in these tumors will be evaluated. This information may also be useful in early detection of thyroid carcinoma in patients at high risk for this disease due to radiation exposure from occupational or therapeutic sources.

We are completing a study on the prognostic value of DNA content analysis in a group of lymph node negative breast carcinomas. This is a controversial area of research with many studies complicated by differences in the population studied and treatment received. Our patients are a homogeneous population from Argentina that have all been treated by the same clinician. This test is currently being used by oncologists in evaluation of breast cancer for adjuvant chemotherapy to prevent metastatic disease. Further study is necessary to clarify the prognostic value of this test.

Major Findings

1. DNA content (ploidy and S-phase) of gastrinomas in Zollinger-Ellison patients correlates independently with the extent of disease.
2. Multiple stem line aneuploid and high S-phase gastrinoma tumors appear to behave aggressively in Zollinger-Ellison patients.
3. Single aneuploid and low S-phase gastrinoma tumors appear to be indolent in Zollinger-Ellison patients.

Publications:

Metz DC, Kuchnio M, Fraker DL, Venzon DJ, Jaffe G, Jensen RT, Stetler-Stevenson M. Flow cytometry and Zollinger-Ellison syndrome: relationship to clinical course. Gastroenterology (in press)



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

PROJECT NUMBER

Z01 CB 09373-02 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

TIMP-1 Expression by Normal Lymphocytes and in Lymphoid Neoplasms

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

PI:	M. Stetler-Stevenson	Medical Officer	LP NCI
OTHER:	K. Ptaszynski	Visiting Fellow	LP NCI
	S. Sebers	Medical Technologist	LP NCI
	J. McClanahan	Medical Technologist	LP NCI

COOPERATING UNITS (if any)

Dr. William Stetler-Stevenson, Laboratory of Pathology, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.75

PROFESSIONAL:

1.75

OTHER:

1

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.)

We have studied the expression of TIMP-1 in a series of Burkitt cell lines. Burkitt's lymphoma, common in AIDS patients, is an aggressive neoplasm without effective therapy in the immunocompromised host. Northern blot analysis reveals that TIMP-1 is expressed at the RNA level by 4 out of 9 lines studied. Cell lines from both African and American Burkitt's express TIMP-1, as do Burkitt cells with t(8;14) and t(8;22) translocations. All of the Burkitt cell lines studied with demonstrable TIMP-1 RNA transcripts secreted TIMP-1 protein (as determined by Western blot analysis) into the culture media. TIMP-1 expression does not correlate with EBV involvement, p53 mutations, or levels of myc expression in these lines. The cell lines that express TIMP-1 have a higher rate of cell proliferation *in vitro* compared to those negative for TIMP-1 transcripts. We are purifying TIMP-1 protein and intend to study the effect of TIMP-1 on growth kinetics *in vitro*. The TIMP-1 expressing Burkitt cell lines are also more tumorigenic and invasive in the nude mouse model. Invasion of skin and nerve is seen in only in TIMP-1 expressing lines. This aggressive behavior may be due to high growth rate, enhanced invasive behavior or a combination of the two. We are currently studying four cell lines (two expressing TIMP-1 and two negative) in invasion assays to assess invasive behavior. We plan to infect TIMP-1 negative cell lines with a retroviral construct containing TIMP-1 to cause TIMP-1 expression in order to study the effect of TIMP-1 on growth rate and invasiveness.

In four of the Burkitt cell lines studied, Gelatinase B activity can be demonstrated by zymogram but not by Northern blot analysis. TIMP-1 and Gelatinase B coexpression is seen in only one cell line, with very low enzyme expression noted. The remainder of the Gelatinase B activity was demonstrated in TIMP-1 negative cell lines. Gelatinase A expression was not observed in any Burkitt cell lines. If TIMP-1 functions in Burkitt's lymphoma by blocking gelatinase activity, we would have expected to be able to demonstrate gelatinase expression by Northern blot or zymogram in the TIMP-1 positive cell lines. As gelatinase expression may occur in these cells in response to the extracellular matrix, we are studying the expression of Gelatinase A and Gelatinase B in Burkitt cell tumors grown in nude mice.

Major Findings:

1. TIMP-1 is expressed in Burkitt lymphoma cell lines and is associated with increased proliferation rate *in vitro*.
2. TIMP-1 expression and secretion is associated with an increase in tumorigenic and invasive behavior in the nude mouse model.
3. TIMP-1 expression is an independent factor in Burkitt cell lines and is not associated with p53 mutations, EBV or c-muc expression.
4. Gelatinase A or B expression is not associated with TIMP-1 expression in Burkitt cell lines.

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

PROJECT NUMBER

Z01 CB 09144-09 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Levens	Chief, Gene Regulation Section	LP NCI
OTHER:	R. Duncan	IRTA Fellow	LP NCI
	E. Alesse	Visiting Fellow	LP NCI
	E. Michelotti	Biotechnology Fellow	LP NCI
	T. Davis-Smith	Biotechnology Fellow	LP NCI
	G. Michelotti	Biotechnology Fellow	LP NCI
	T. Tomonaga	Visiting 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 STAFF YEARS:

7.5

PROFESSIONAL:

7.5

OTHER:

0

CHECK APPROPRIATE BOXES!

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

B

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

We have been studying three regulatory elements of the human c-myc proto-oncogene and the proteins which bind to them.

1) 1.5 kb upstream of promoter P1 resides a cell-type and differentiation specific positive cis-element. A novel protein binding to this element has been purified and subjected to proteolytic degradation and partial sequence determination. The information thus obtained allowed the cloning of a gene encoding a novel DNA binding protein. This protein possesses a structure comprised of alternating amphiphathic helices (five) and repeating units (four). The minimal sequence specific DNA binding domain is composed of two helices and two repeats. Analysis of the expression of this factor reveal it to be tissue specific and highly regulated. Functional characterization of the protein is ongoing. This protein binds specifically to the FUSE element in either single or double-stranded form.

2) 100-150 bp upstream of P1 a complex set of trans-factors binds to a cytidine-rich element repeated five times. Some of the factors which interact with this element possess the ability to recognize specific single-stranded sequences. One of the pyrimidine-rich strand binding proteins is hnRNP protein K. *In vitro* and *in vivo* evidence strongly implicates hnRNP protein K as a trans-activator of this site. Additional proteins interact with the purine strand. The purine strand binding factors possess several unusual properties which correlate well with the activity of this element in *in vitro* transcription systems. One of these factors has been identified as an altered form of a zinc finger protein. The purification of the remaining factors with an aim of cloning their genes is in progress.

3) Approximately 1 kb downstream of c-myc promoter P1 is an element which has been found to be mutated frequently in Burkitt lymphoma. Several proteins have been shown by cross-linking or binding with SDS-PAGE purified and renatured polypeptides to interact with this element. Some of these proteins possess unusual properties which suggest interesting regulatory mechanisms for controlling c-myc expression. Based upon newly derived peptide sequence, the identification of one of these factors as a new member of a known family of DNA binding proteins has been made. Cloning of this factor is in progress.

The c-myc gene has multiple cis- and trans-elements both upstream and downstream of the major c-myc promoters P1 and P2. Three elements, originally described in the section, are being studied extensively. First, because cessation of c-myc transcriptional initiation has been shown to occur during pharmacologically induced differentiation of monomyelocytic leukemia cell lines and because this event appears to be a prerequisite for differentiation, experiments to identify a differentiation inducible repressor or a differentiation repressible activator were performed. Modulation of a factor as detected by loss of binding activity to a site 1500 bp upstream of promoter P1 was noted. The precise binding site was defined by deletional and mutational analysis. Functional transfection studies have indicated that this binding site serves as a positive element in undifferentiated leukemia cells. Following differentiation, the far upstream element, designated FUSE, ceases to stimulate c-myc expression. A 75 kD protein binding to the FUSE was purified and micro-sequence analysis allowed the cloning of a gene encoding the FUSE binding protein (FBP). The FBP protein possesses a novel structure including a new DNA binding motif. This motif appears to confer single strand, sequence specific recognition properties. The FBP gene stimulates c-myc promoter mediated expression and deletion of the FUSE element diminishes this stimulation. Expression of FBP itself is regulated, being shut off during differentiation. Screening for genes related to FBP has revealed the existence of other highly related proteins suggesting that FBP may be the prototype for a new family of gene regulatory proteins.

Previously, a cis-element was identified in intron 1 of the human c-myc gene and was demonstrated to bind a nuclear protein. This element is mutated in most Burkitt's lymphomas. We have extended these investigations by identifying a 140 kD phosphoprotein responsible for this binding activity. Importantly, phosphorylation appears to be necessary for strong binding to the myc intron sequence. The 140 kDa protein has been purified from HeLa cells and the sequence of several internal tryptic peptides has been determined, revealing the myc-intron binding protein to be a new member of a known family of DNA binding proteins. Cloning of the full-length cDNA to allow *in vivo* and *in vitro* studies of protein structure and function are in progress. Additionally, a second protein component of the specific DNA-binding complex has been identified as a protein of approximately 35 kD. The role of this second factor in c-myc regulation is under investigation. Functional analysis of the binding for this complex is in progress. The site serves as a positive cis-element in HeLa cells.

One hundred bases upstream of the c-myc promoter is an element composed of multiple repeats of the sequence CCCTCCCCA. This element stimulates expression from P2 and is essential for expression from P1. A complex array of factors interacts with site. A protein which binds to the CT-element in a highly sequence specific fashion was purified. Surprisingly, this factor also displays a marked preference for interacting with one of the two strands of the CT-element. Protein sequence analysis revealed this protein to be an isoform of hnRNP protein K; hnRNP protein K is a highly atypical hnRNP protein. It lacks any well described RNA binding motif and has no homology to any known RNA binding protein. Surprisingly, hnRNP protein K clearly possesses the same DNA binding motif present in FBP. Investigations are commencing to study the roles and interaction of FBP and hnRNP protein K as they effect c-myc expression.

The identification of the proteins which regulate c-myc expression and the elucidation of the mechanisms of their action is a basic science problem with ramifications at several levels for the diagnosis, treatment and prevention of human disease. As c-myc expression is modulated in a variety of physiologic and pathologic conditions, if the role of the factors regulating c-myc individually and ensemble were defined then: 1) individual trans-elements could be identified as significant targets for potential genetic aberrations associated with inherited disease or somatic mutations. In the former instance genetic counseling could be useful in disease prevention, in the latter case use of nucleic acid markers could be applied for early diagnosis and treatment. 2) The regulators of c-myc expression are themselves highly regulated. If the hierarchy of inputs were established and if the diverse inputs were linked to known signal transduction pathways, then many possibilities for pharmacologic or biologic manipulation exist. The ability to predict and control c-myc expression would provide extraordinary influence over cell growth and death.

#### Major Findings:

- 1) A protein with a novel structure interacts with single strands of the FUSE segment of the human c-myc gene. This protein stimulates c-myc promoter driven expression in a FUSE dependent manner.
- 2) There are several other genes encoding proteins closely related to that mentioned above.
- 3) A site in intron 1 of the human c-myc gene binds a 140 kD protein, a new member of a known family, based upon internal peptide sequences. This site behaves as a positive cis-element in Hela cells.
- 4) hnRNP protein K behaves as a transcription factor stimulating expression from a site 100 to 140 bp upstream of P1. Several other factors have been identified which interact with this element.

#### Publications:

Takimoto M, Tomonaga T, Matunis M, Avigan M, Krutzsch H, Deyfuss G, Levens D. hnRNP protein K binds to the c-myc promoter *in vitro*. J Biol Chem (in press)

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

PROJECT NUMBER

Z01 CB 09168-06 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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. Levens	Chief, Gene Regulation Section	LP NCI
OTHER:	K. Gardner	Med. Staff Fellow/Sr. Staff Fellow	LP NCI
	H. Yasui	Guest Researcher	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS

3.5

PROFESSIONAL:

2.5

OTHER:

1.0

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 (a1) Minors  
 (a2) Interviews

B

SUMMARY OF WORK (Use standard unindented 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 binds with reduced specificity to DNA, but upon forming a complex with the first component, confers greatly enhanced 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.

The first component of the complex has been identified as a modified form of the jun-d protein. The modification, thus far detected only in T-cells, causes the mobility of the jun-d to increase both in SDS-polyacrylamide gels and in DNA binding electrophoretic mobility shift assays. The nature of the modification is under investigation. In addition, it appears that a population of jun-d exists in T-cells which is relatively inactive with the GALV-AP1 site. Modified jun-d, but not recombinant jun-d interacts with a second component, a protein of approximately 20,000 MW, which augments binding to AP1 sites. Importantly this second protein, termed activator, may play an important role in T-cell activation, as it is a component of NFAT (nuclear factor of activated T-cells) critical for IL-2 induction.

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. Although this element contains an AP1 site, the factors which bind this site in T cells display properties distinct from those described for members of the fos/jun family.

Factors interacting with the enhancer of the gibbon-ape leukemia virus are important in T-cell activation. Studies of the GALV enhancer have led to the study of jun-d and associated factors in T-cell activation. The GALV binding complex has been shown to be composed of multiple components which pre-exist in resting T cells and are activated by a post-translational mechanism. jun-d seems to be the most important player of the AP1 family during the earliest stages of activation. Protein modification seems necessary for producing a functional jun-d protein; these changes include phosphorylation, but accumulating evidence suggests that other covalent changes are required as well.

A second component has been purified which binds cooperatively with jun-d protein showing a preference for natural over recombinant jun-d. This 23 kDa protein, designated "activator", stimulated jun-d binding more than one-hundred fold and stimulated GALV-AP1 site directed transcription, *in vitro*. Importantly, considerable evidence indicates that this same factor is part of the NFAT (nuclear factor of activated T cells) complex. NFAT is a multi-component complex which plays a crucial role in directing the T-cell specific stimulation of IL-2 gene expression. Importantly, NFAT has been shown to contain an as yet undefined member(s) of the AP1 family, thus providing a unifying link for activator specificity. The activator displays novel properties which suggest that it should play an important role in T-cell function. Sufficient activator has been purified for protein sequencing and the section plans on obtaining a full-length cDNA as soon as suitable peptide sequence is available.

This project in the Gene Regulation Section has 1) identified a modified form of jun-d as an important player early in T-cell activation and identified a new protein designated "activator" which acts cooperatively with transcriptional activators at AP1 and NFAT sites, possibly at NF kappa B sites as well. As these elements are also critical components of the IL-2 induction machinery, it seems likely that the activator will participate in an important fashion in T-cell activation. Knowledge of activator structure, function and regulation may allow us to evaluate its function in normal and pathologic immune conditions potentially providing a marker for disease, genetic or acquired, and manipulation of activator may allow therapeutic modulation.

Major Findings:

- 1) jun-d is post-translationally modified during early stages of T-cell activation.
- 2) A 23 kDa protein activates jun-d binding to AP1 sites and also stimulates binding of NFAT.
- 3) The 23 kDa protein stimulates transcription *in vitro* from NFAT and AP1 sites.



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

PROJECT NUMBER

Z01 CB 09170-06 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Identification of Genes Regulating the Development of Embryonic Limb Buds

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

PI:	S. Mackem	Expert	LP NCI
OTHER:	S. Aguanno	Visiting Fellow	LP NCI
	R. DeSanto	General Fellow	LP NCI
	V. Knezevic	Visiting Fellow	LP NCI

COOPERATING UNITS (if any)

(research collaborators)-K. Mahon, LMGD, NICHD; S. Hughes, FCRC; K. Schughart, Max Planck Inst. of Immunol., Freiburg, Germany; C. Tickle, Dept. of Anat., Univ. College of London, U.K.; J. Fallon, Dept. of Anat., Univ. of Wisconsin

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Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

3.5

OTHER:

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- (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.)

The identification of genes necessary for establishing pattern formation during morphogenesis and the study of their regulation are relevant to many aspects of vertebrate biology. Many key processes in morphogenesis, including responses to trophic stimuli, cell-cell interactions, migration, differential proliferation, programmed cell death, etc., are recapitulated in a pathologic manner during oncogenesis. So, an understanding of processes active during morphogenesis and their regulation will ultimately contribute to a better understanding of mechanisms of oncogenesis and metastasis, and may provide insight into cancer prevention and treatment strategies. Chick limb development is an attractive system for studying the molecular basis of pattern formation because critical events at the level of tissue/cellular interactions have been well characterized and are very similar to those in mammals, and this system is readily amenable to biochemical and molecular analysis as well as microsurgical manipulation.

The aim of this project is to isolate genes that regulate morphogenesis in the chick embryo limb bud. Two approaches are being developed: 1) the generation of subtracted cDNA libraries enriched for potential regulatory and induced genes; and 2) the identification of related/new members of conserved gene families that have been implicated in developmental regulatory processes in other systems. Two novel members of the homeobox gene family have been identified which show spatially restricted expression domains within the limb bud that suggest roles in regulating pattern formation along the anterior-posterior (A-P) and the proximodistal (P-D) axes of the limb. One of these genes is also expressed in the very early embryo during gastrulation, as well as the limb bud, and may regulate the determination of positional identity along the primary embryonic axis, as well as the limb P-D axis. Studies are underway to elucidate the function of these regulatory genes, using both molecular genetic and biochemical approaches.

### Major Findings:

Oriented cDNA libraries have been generated from limb bud mRNA populations, for use in both general screening and for performing library-based subtractive hybridizations to enrich for genes involved in establishing morphologic patterns and in regulating the pattern differences between wing and leg that constitute limb-type identity. These include early (stage 17/18) wing, leg and late (stage 21/22) wing, leg libraries. These different stages were chosen as likely to represent times at which signals regulating A-P and P-D patterning are just beginning to be expressed (early) and times at which apparent morphogenetic gradients are well established (late), but prior to the onset of frank tissue differentiation. Subtracted libraries enriched for wing- and for leg-specific sequences have already been generated and screening with wing- and leg-enriched cDNA probes is underway to isolate genes regulating differences in wing/leg pattern formation.

We have used a PCR-based approach employing degenerate oligonucleotide primers for amplification, and subsequent subcloning and sequencing, to identify homeobox genes that are expressed in limb bud mRNA populations. At least 18 different homeobox genes appear to be expressed in chick embryo limb buds. Some of these include genes that have previously been characterized in other vertebrate systems and are known to be expressed in developing and/or regenerating limbs. Several genes appear to be new members of the homeobox family in vertebrates and two of these are selectively expressed predominantly in limb buds during early development. These genes are novel non-Antennapedia homeobox genes with homeodomain sequences of some similarity to *Drosophila Abd-B* (*Hoxd-12*, previously *Ghox 4.7*) and to *Drosophila EMS (L5)* respectively, and we have analyzed their spatiotemporal expression domains during development using *in situ* hybridization techniques on both sectioned embryos and on whole mount embryos.

*Hoxd-12* (*Ghox 4.7*) has been named such because it is homologous to the murine gene. We have found that this gene is expressed in a very posteriorly restricted domain of the early limb bud, suggesting a role in patterning along the A-P axis. Others have shown that manipulations which alter the developmental program of the limb bud to produce mirror image duplication of skeletal elements along the A-P axis will also result in ectopic mirror-image duplication of expression of several of the *Hoxd* (*Hox 4*) cluster genes in the anterior limb bud, supporting the notion that these posteriorly expressed genes regulate the A-P pattern. Using whole mount *in situ* hybridization with several of these chick *Hoxd* cluster genes, we have found that two of the very posteriorly restricted *Hoxd* genes display both quantitative and qualitative expression differences between wing and leg buds in the chick embryo. These differences are not seen in murine embryos and so may be related to modification of the avian wing from the general tetrapod limb pattern for flight. Since some of the posterior elements of the wing are enlarged and dominate its structure, the limb-type differences in chick *Hoxd* expression may also be a reflection of its role in regulating A-P pattern.

Long-term genetic experiments designed to determine the function of the regulatory genes we have characterized are also underway, and include ectopic overexpression as well as ablation of expression of these genes using transgenic technology in mice, and avian retroviral expression vectors in chick embryos. These genetic analyses are already in progress for analyzing the function of the *Hoxd-12* gene. The complete coding sequence as well as sequences containing only selected protein "domains" have been introduced into avian retroviral expression vectors to examine the effects of transient ectopic expression in chick embryos. Similar constructs have been made for introduction as transgenes into mice, using a promoter which gives high level expression primarily in the limb buds of developing embryos (a truncated *HoxB-6* (*Hox 2.2*) promoter) in order to target (and restrict) overexpression of *Hoxd-12* to the developing limb bud. Our preliminary results expressing the full-length *Hoxd-12* protein in the developing limbs of transgenic mice are consistent with a proposed role for this gene in specifying positional information along the A-P axis of the limb; the mice display a phenotype in which the anterior limb skeletal elements are transformed to a posterior-type of morphology.

Biochemical approaches are also being employed to analyze the function of *Hoxd-12*. The cis DNA sequence elements to which the *Hoxd-12* protein binds have been identified. Specific antibodies against the *Hoxd-12* protein have been raised, and these will be useful in the isolation of *in vitro* and also *in vivo* complexes formed between the *Hoxd-12* protein and genomic DNA for the purpose of identifying downstream target genes that *Hoxd-12* regulates. *Hoxd-12*/GST fusion proteins are being employed to identify other proteins with which *Hoxd-12* interacts in limb bud extracts.

The *L5* gene has a highly restricted expression domain along the P-D axis of the limb, which changes with time as elements are progressively specified/determined along this axis. Expression is first seen early (st 19) in the distal limb bud, both in the mesenchyme and overlying ectoderm, particularly the apical ectodermal ridge, which functions to induce orderly limb outgrowth along the P-D axis. At later stages (st 26-28), the expression is localized more proximally, and is restricted to the region of the anterior distal zeugopod (radius or tibia) and proximal autopod (carpals or tarsals). This type of expression is consistent with the known progressive determination of structures along the P-D axis in a proximal to distal sequence and suggests a role for *L5* in the determination of positional identity (for example of the wrist/ankle bony patterns) along the P-D axis. Microsurgical manipulations (apical ridge excisions/grafts; retinoid treatment) are currently underway to analyze the expression pattern of this gene when the developmental program (pattern) is experimentally altered. Our preliminary results indicate that the presence of the apical ectodermal ridge is absolutely required for the early *L5* expression in the limb bud. However, at a later stage of limb development (ie. a time at which the wrist/ankle patterns have already been definitely determined under the influence of the apical ridge), the presence of the ridge is no longer necessary to maintain *L5* expression. These findings are also consistent with a role for *L5* in the determination of positional identity in the presumptive wrist/ankle regions.

Interestingly, *L5* is also expressed in two other very restricted locations in the early embryo; the anlage of the pineal gland, and in Hensen's node and the notochord arising from it during gastrulation. Hensen's node in the chick (and mouse) is thought to be the equivalent of Spemann's organizer in xenopus (dorsal lip of the blastopore), and induces formation of the embryonic axis during gastrulation. The notochord, which arises from Hensen's node anteriorly, is critical for inducing the neural tube and is also thought to play a role in somitogenesis, the process by which segmented somites appear in an orderly temporal sequence from anterior to posterior along the strips of paraxial mesoderm. During somitogenesis, the expression of *L5* within the notochord recedes posteriorly concomitant with the adjacent segmentation of paraxial mesoderm into discrete somites. Following somitogenesis, *L5* expression in the notochord is restricted to the very posterior tip. Notably, in this region the adjacent paraxial mesoderm never becomes segmented in the chick. This pattern of expression suggests that *L5* plays a role in regulating somitogenesis along the embryonic A-P axis.

We are also beginning experiments intended to alter the normal expression pattern of *L5* in chick embryos using retroviral expression vectors. To study effects of altering the very early expression of *L5* during and just after gastrulation, we are developing techniques to inject antisense oligonucleotides under the blastoderm of the unincubated chick embryo. We are also cloning the murine homologue of this gene to facilitate the introduction of expressed transgenes and/or 'knock-out' constructs into mice to evaluate function.

#### Publications:

Mackem S, Mahon K. *Glox 4.7*: A chick homeobox gene expressed primarily in limb buds with limb-type differences in expression. *Development* 1991;112:791-806.

Mackem S, Ranson M, Mahon KA: Limb-type differences in expression domains of certain chick *Hox-4* genes and relationship to pattern modification for flight. In: Fallon J, Goetinck PF, Kelley RO, eds. *Proceedings of the Fourth International Conference on Limb Development and Regeneration*. New York: Wiley-Liss, 1993 (in press)

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

PROJECT NUMBER

Z01 CB 09171-10 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

The Regulation of Lymphocyte Proliferation

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

PI:	K. Kelly	Senior Staff Fellow	LP NCI
OTHER:	K. Smith	Microbiologist	LP NCI
	J. Gray	Fogarty 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 STAFF YEARS:

2.3

PROFESSIONAL:

1.3

OTHER:

1.0

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

B

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

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 currently are investigating 2 induced genes that encode integral membrane proteins. Activation-induced changes in cell surface proteins resulting from a primary stimulus play a particularly important role in regulating downstream proliferative and differentiative responses. Important events mediated at the cell surface include the binding of soluble factors and interactions with other cells and extracellular matrix. 276 encodes a protein of approximately 87 kD. The carboxy half of the protein contains seven membrane spanning domains such as those conserved in the class of receptors that bind heterotrimeric G proteins, and an amino-terminal large extracellular domain contains EGF-like repeats and an RGD sequence. The large extracellular domain is co-translationally proteolytically cleaved from the membrane spanning region into an approximately 50 kD protein backbone that remains associated with the cell surface. The structure of the extracellular domain suggests a role in cell/cell or cell/matrix interactions. An interesting possibility is that the extracellular domain of 276 is involved in ligand recognition and serves as a regulatory subunit to the heterotrimeric G protein-coupled receptor. 154 encodes a 158 amino acid type 1b membrane protein with a single amino terminal hydrophobic domain and the body of the protein projecting into the cytoplasm. Endogenously-synthesized 154 co-localizes with transferrin receptor in cytoplasmic vesicles, and therefore may be a recycling surface protein or an integral membrane protein involved in vesicle trafficking.

**Major Findings:**

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. Activation-induced changes in cell surface proteins resulting from a primary stimulus play a particularly important role in regulating downstream proliferative and differentiative responses. Important events mediated at the cell surface include the binding of soluble factors and interactions with other cells and extracellular matrix. Two mitogen-induced genes (154 and 276) encode integral membrane proteins and will be discussed here.

The deduced protein sequence for 276 codes for a protein of approximately 87 kD. The carboxy half of the protein demonstrates the conserved structure of and homology to the class of receptors that bind heterotrimeric G proteins, and an amino-terminal large extracellular domain contains EGF-like repeats that are most highly related to the basement membrane protein, nidogen, and the microfibrillar protein, fibrillin. EGF repeats are most likely involved in protein/protein interactions such as receptor/ligand or receptor/extracellular matrix binding. A large extracellular domain is a highly unusual feature for G-coupled protein receptors that most often display less than 100 amino acids on the extracellular surface. Pulse-chase experiments demonstrate proteolytic processing of the 87 kD peptide backbone structure. 276 is cleaved in transfected COS-7 cells to generate an N-linked deglycosylated peptide of about 50 kD (the glycosylated form is about 68 kD), and in activated T cells to yield 3 N-linked deglycosylated products between 46 and 65 kD. The 3 products in T cells may represent distinct alternative primary structures or differences in O-linked glycosylation. Whole cell iodination studies have shown that the large, extracellular domain stays associated with the plasma membrane despite having no transmembrane region. We are producing a soluble form of the extracellular domain in order to assay its interaction with extracellular matrix or other cell surface-associated structures. An interesting possibility is that the extracellular domain is involved in ligand recognition and serves as a regulatory subunit to the heterotrimeric G protein-coupled receptor.

154 encodes a 158 amino acid peptide with a hydrophobic leader sequence but no other transmembrane domain. The hydrophobic leader does not appear to be cleaved. Previous studies demonstrated that 154 is not secreted. Endogenously-expressed 154 protein in the human myeloma cell line U266 has been co-localized with transferrin receptor to cytoplasmic vesicles. The membrane association of 154 with vesicles strongly suggests that it is an unusual type 1b membrane protein with a single membrane spanning domain and an orientation of the carboxy terminus in the cytoplasm. It is not clear whether 154 is being recycled from the plasma membrane as is true for transferrin receptor or whether 154 may have a role to play in vesicle trafficking.

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PROJECT NUMBER

Z01 CB 09357-03 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

GEM: A Mitogen-Inducible RAS-Related Protein

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

PI:	K. Kelly	Senior Staff Fellow	LP NCI
OTHER:	P. Davis	Technician	LP NCI
	T. Santoro	Guest Researcher	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 (a1) Minors  
 (a2) Interviews

B

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

A gene has been isolated from an activated human T cell cDNA library that encodes a novel, 35 kD phosphorylated GTP-binding protein (designated GEM) that demonstrates approximately 20 percent homology to c-Ha-ras. GEM contains approximately 10 kD of unique ser/thr rich sequence on the amino terminus and 7 kD of unique sequence, rich in basic amino acids, on the carboxy terminus. GEM has been shown to preferentially bind GTP over ATP. GEM expression is highly regulated. GEM RNA and protein are transiently expressed in mid-G1 following mitogenic activation of T cells and fibroblasts. GEM RNA is expressed in several different murine tissues of hematopoietic and non-hematopoietic origins. Transfected and endogenously expressed GEM has been shown to localize to the inner face of the plasma membrane, and to cytoplasmic vesicles in neuronal cells. In addition to the GTP-binding domain, the carboxy but not amino terminus is required for membrane localization. The GTP-binding property of GEM, in addition to its identification as a phosphorylation substrate and membrane-associated protein, suggest a role in signal transduction.

Major Findings:

A gene has been isolated from an activated human T cell cDNA library that encodes a novel, 35 kD GTP-binding protein (designated GEM) that demonstrates approximately 20 percent homology to c-Ha-ras. GEM contains approximately 10 kD of unique ser/thr rich sequence on the amino terminus and 7 kD of unique sequence, rich in basic amino acids, on the carboxy terminus. GEM has been shown to preferentially bind GTP over ATP. GEM expression is highly regulated. GEM RNA and protein are transiently expressed in mid-G1 following mitogenic activation of T cells and fibroblasts. GEM RNA is expressed in several different murine tissues of hematopoietic and non-hematopoietic origins.

We have used a monoclonal anti-GEM antibody and immunofluorescence analyses to localize GEM in endogenously-expressing and transfected cell lines. GEM localizes to the plasma membrane in transfected CV-1 or HeLa cells. Because GEM has no transmembrane region, it must be associated with the inner face of the membrane as is true for K-ras and H-ras. Transfections with constructs of GEM deleted in the amino or carboxy terminal regions, outside the ras homology region, have shown that the carboxy terminus but not the amino terminus is required for membrane localization. However, GEM does not contain an isoprenylation consensus sequence in the carboxy terminus. We are currently investigating the mechanism (most likely involving lipid modification) whereby GEM associates with membranes. In the human embryonic carcinoma cell line NTERA-2, endogenously synthesized GEM localizes to the plasma membrane. Following differentiation of NTERA-2 cells with retinoic acid to immature neurons, GEM protein increases in abundance and localizes not only to the plasma membrane, but also to vesicles surrounding the nucleus. Thus, in certain cell types, GEM may be involved in secretion. We are currently investigating GEM expression in rat PC12 pheochromocytoma cells that serve as a widely used model for neuronal differentiation.

We have cloned the human and murine genomic loci for GEM in order to establish the exonic structure of the coding region (necessary information for genetic manipulations) and to isolate the regulatory region of this gene. The GEM coding sequence is contained on 5 exons. Further mapping studies are underway in preparation for making vectors to be used in gene knockout experiments.

The transcriptional regulation of GEM is under investigation. Using a human genomic clone, the transcription start site of GEM has been mapped by primer extension. A 1732 bp fragment that terminates just 3' to the transcription initiation site was cloned into a promoterless chloramphenicol acetyl transferase (CAT) reporter plasmid. Transfection experiments are being conducted on hematopoietic (Jurkat) and non-hematopoietic (NTERA-2 and NIH-3T3) cells in which GEM transcription is regulated by external signals. Using exonuclease III digestion of the regulatory region, clones have been generated extending 1300, 1086, 587, 376, and 64 bp 5' of the promoter. These clones will be used to define positive and negative elements required for constitutive and inducible expression of GEM.



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

PROJECT NUMBER

Z01 CB 09358-03 LP

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Characterization of a Mitogen-Inducible Tyrosine Phosphatase, PAC-1

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

PI:	K. Kelly	Senior Staff Fellow	LP NCI
OTHER:	P. Rohan	Biotechnology Fellow	LP NCI
	P. Jensen	Technician	LP NCI
	Y. Ward	Fogarty 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 STAFF YEARS:

3.4

PROFESSIONAL:

2.4

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.)

We have cloned a mitogen-induced gene from human peripheral blood T cells that encodes a 32 kD protein phosphotyrosine phosphatase (designated PAC). PAC is a member of a newly emerging family of PTPases, the VH1 family. Several VH1 family members have interesting properties, including highly regulated expression and phosphatase activity against both phosphorylated ser/thr and tyr residues. PAC protein is observed transiently in mid-G1 following activation via signals that lead to cell division and differentiation. PAC RNA expression is limited to mature and developing hematopoietic tissues (spleen, thymus, and bone marrow). PAC has been localized to the nucleus in transfected COS-7 cells and mitogen-activated T cells. We are currently characterizing PAC phosphatase activity and evaluating several potential phosphorylated nuclear protein substrates. The expression properties of PAC suggest a role in cellular signalling.

Major Findings:

We have cloned a mitogen-induced gene from human peripheral blood T cells that encodes a protein phosphotyrosine phosphatase (PTPase). This gene codes for a 32 kD protein, designated PAC, for phosphatase of activated cells. PAC RNA and protein expression is highly regulated; PAC protein is observed transiently in mid-G1 following activation via signals that lead to cell division and differentiation. PAC RNA expression is limited to mature and developing hematopoietic tissues (spleen, thymus, and bone marrow). The expression properties of PAC suggest a role in cellular signalling.

PAC is part of an emerging family of PTPases. PAC is similar to a phosphatase induced by serum or heat shock in fibroblasts (CL100), a phosphatase in yeast whose abundance is regulated by nitrogen (YVH1), a phosphatase from human fibroblasts (VHR), and a vaccinia virus-encoded phosphatase (VH1). This family of phosphatases, referred to as the VH1 family, displays a 100 amino acid region of similarity that includes the active site of the enzymes. Other than the enzymatic signature sequence, the VH1 family is structurally different from several other known related PTPases exemplified by PTP-1b. Several VH1 family members have been shown to be dual specific phosphatases, exhibiting phosphotyrosine and phosphoserine/threonine activity. Southern blot analyses with probes that encode different structural domains of PAC reveal one additional gene, highly related to PAC over its entire length, and several other genes that are homologous in the region surrounding the active site. Cloning of related genes from hematopoietic cDNA libraries is in progress.

Utilizing immunohistochemistry, PAC was shown to localize to the nucleus in transfected COS-7 cells and in mitogen-activated T cells. Similarly, we have shown that transfected CL100 localizes to the nucleus in COS-7 cells. A consensus bipartite nuclear localization signal (NL) exists in PAC within the VH1 family homology region. We are currently producing mutated versions of the bipartite NL consensus to test whether it is utilized as a nuclear localization signal and, if so, which conserved amino acids are required.

In order to address the substrate specificity of PAC, we have produced recombinant bacterial proteins that encode glutathione-S-transferase fused to 18 kD of PAC that contains either the active enzymatic site or a point mutation that destroys enzymatic activity. The purified, wild-type recombinant protein has activity toward p-nitrophenol phosphate (pNPP), but not toward a tyrosine phosphorylated peptide (RAYTIDE), a commonly used substrate to assay PTPases. Such results suggest either a high degree of substrate specificity, or alternatively, a predominantly inactive tertiary structure of the protein produced in bacteria. To address the latter possibility, we have begun purifying over-expressed PAC from transfected COS cells. Because PAC is most likely a dual specific phosphatase, it seems probable that the natural substrate will be phosphorylated on both serine or threonine and tyrosine. Therefore, we have begun to investigate substrates whose activities are modulated by both tyrosine and ser/thr phosphorylation. A nuclear substrate of this type is MAP kinase, an important regulatory protein in mediating signal transduction between the cell membrane and the nucleus. MAPK is currently being investigated as a potential substrate for PAC.

Publications:

Rohan PJ, Davis P, Moskaluk C, Kearns M, Krutzsch H, Siebenlist U, Kelly K.  
Pac-1: A mitogen-induced nuclear protein tyrosine phosphatase. Science  
1993;259:1763-6.



SUMMARY STATEMENT  
ANNUAL REPORT  
DERMATOLOGY BRANCH  
DCBDC, NCI

October 1, 1992 through September 30, 1993

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 2,000 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 (Dr. Stephen Katz):

We have continued our studies of the immunological functions of cells of the epidermis. During the past year our studies have continued to focus on the very earliest events which occur after skin is exposed to haptens and other chemicals, and have found that within 24 hours there is "activation" of Langerhans cells as demonstrated by their expressing greatly increased amounts of class II MHC on their surfaces, as well as their becoming much more potent antigen presenting cells than are "unstimulated" Langerhans cells. As the earliest manifestation of LC activation is the accumulation of increased amounts of IL-1 $\beta$  mRNA in LC within 15 min after exposure to contact allergens, we assessed the functional role of IL-1 $\beta$ . We found that IL-1 $\beta$  mimics the effects of allergens on LC class II MHC antigen expression, accessory cell activity and mobilization and simulates the effects of contact allergens on LC- and keratinocyte-derived cytokine mRNA signals. In aggregate, our studies show that IL-1 $\beta$  plays an essential role in the initiation of primary cutaneous responses. We have also demonstrated that IL-10 mRNA and protein production by keratinocytes is enhanced after application of contact allergens. Recently, we have shown that IL-10 inhibits the ability of Langerhans cells to present antigens to Th1 type helper T cell clones. No effect of IL-10 treatment of Langerhans cells was seen when Th2 type helper T cell clones were used.

During this past year we have initiated studies that assess the phenotypic and functional characteristics of epidermal Langerhans cells in the skin of patients with AIDS. In our first cohort of patients, we found that Langerhans cells function at least as well as peripheral blood monocytes in the activation of T cells. We are continuing these studies and attempting to use, as responder cells, T cells from nonaffected identical twins. As well, we are assessing the ability of HIV to infect Langerhans cells from HIV-infected patients as well as normal human epidermal cells in vitro. These studies will hopefully provide insight into the role of skin as an initiator of inflammatory and perhaps neoplastic lesions in skin of HIV-infected patients.

Regulation of Cutaneous Accessory Cell Activity in Health and Disease  
(Dr. Mark Udey):

A major focus of this laboratory has been the study of cell-surface adhesion molecules important in Langerhans cell (LC)-T cell activation. In the past year a major effort has been directed towards the study of cadherins expressed by leukocytes. Cadherins comprise a growing supergene family of calcium-dependent homophilic adhesion molecules known to be involved in embryogenesis and in the maintenance of structural integrity in epithelia and the nervous system. Cadherins had not been identified on leukocytes at the time these studies were begun. It was determined that fresh murine BALB/c LC and keratinocytes KC express similar levels of E-cadherin, a cadherin initially described in epithelia. LC appear to actively synthesize E-cadherin, because LC contain E-cadherin mRNA. E-cadherin mediates adhesion of LC to E-cadherin-transfected fibroblasts and KC in vitro, and adhesion of LC to KC can be selectively inhibited by anti-E-cadherin monoclonal antibodies. Cultured LC, cells that may represent the in vitro equivalent of LC that have migrated from epidermis to regional lymph nodes, express lower levels of E-cadherin and adhere less avidly to KC.

Dendritic cells (DC) from various lymphoid tissues were subsequently surveyed for E-cadherin expression. Splenic DC and DC prepared from gut-associated lymph nodes (LN) do not express E-cadherin. Results of two color flow cytometry studies suggest that skin-associated LN DC uniformly express low levels of E-cadherin, however. These latter cells may correspond to LC that have migrated from epidermis to regional LN after epicutaneous exposure to antigen. A subpopulation of thymic DC may also express low levels of E-cadherin. Methods for culturing DC from blood and bone marrow have recently been described. DC from the blood of cyclophosphamide-pretreated mice and from the bone marrow of normal animals have also been propagated. Blood and bone marrow DC express various DC surface antigens, and also transiently express E-cadherin in vitro. We propose that E-cadherin is a differentiation antigen expressed by LC, cells that may be derived from LC (skin-associated LN DC), cells that may give rise to LC (blood and bone marrow DC) and cells that may be related to LC (thymic DC). It also seems likely that E-cadherin plays an important role in the localization of LC in epidermis, but this has not been formally tested.

Molecular Basis of Autoimmune Skin Diseases (Dr. John Stanley):

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. cDNA cloning of pemphigus vulgaris antigen (PVA) indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules. PVA is most closely related to desmoglein I, which is also in the cadherin supergene family, and is pemphigus foliaceus antigen. PVA and desmoglein I have very similar structures. Each have repeating, homologous extracellular domains, a transmembrane segment, and similar cytoplasmic domains. Pemphigus vulgaris patients' sera have antibodies that bind the

amino-terminal extracellular domain of PVA, a region of cadherins thought to be important for their function of homophilic binding. IgG from these patients' sera, affinity purified on this domain, is capable of causing the pathology of pemphigus vulgaris blisters (i.e. loss of epidermal cell adhesion) in a well-established neonatal mouse model of disease. In this model, these affinity purified antibodies localize to the core of separating desmosomes (cell-cell adhesion junctions), as determined by immunogold electron microscopy.

A chimeric cDNA that encodes the extracellular domains of PVA and the transmembrane and cytoplasmic domains of E-cadherin has been constructed and used for transient and permanent transfections of eukaryotic cells. Immunoblotting, immunofluorescence, and immunoprecipitation studies of these cells indicates that the cDNA is expressed as a transmembrane protein whose cytoplasmic domain binds catenins, just as the cytoplasmic domain of whole E-cadherin. These transfected cells will be used to further study the biological function of PVA.

#### Therapy of Skin Cancer and Disorders of Keratinization (Dr. John DiGiovanna):

The goal of these studies is to explore the efficacy, toxicity, and mechanisms of action of new treatments for dermatologic diseases with particular emphasis on skin cancer and disorders of keratinization. Studies directed at skin cancer treatment and prevention are continuing. The effectiveness of high-dose oral isotretinoin as a chemopreventive agent in patients with high rates of skin cancer formation has been demonstrated. Low doses of isotretinoin were not very effective preventive agents. Patients are now being treated with intermediate doses to achieve adequate chemoprevention with minimal toxicity.

Dr. DiGiovanna is also actively collaborating with several groups to determine whether genetic linkage exists between certain heritable skin diseases and gene clusters. In collaboration with Allen Bale at Yale, he has identified linkage of the nevoid basal cell carcinoma syndrome gene to 9q31. In tumors, loss of heterozygosity in that region suggests that a mutation in a tumor suppressor gene is the probable cause of the disease. Linkage analysis is being pursued for fine mapping of the region. In collaboration with Drs. S. Bale and P. Steinert (Laboratory of Skin Biology - NIAMS), he was the first to identify linkage of the epidermolytic hyperkeratosis gene to the type II keratin gene cluster on chromosome 12q. They have now identified mutations in 11 families and have identified 7 distinct clinical phenotypes. The correlation between these mutations and clinical phenotypes should lead to a better understanding of the relationship between keratin structure and function.

#### Studies of DNA Repair in Normal Human Cells from Patients with Xeroderma Pigmentosum and Neurodegenerative Disorders (Dr. Jay Robbins):

Studies in this laboratory are designed to elucidate the role of DNA repair processes in carcinogenesis and in neurodegeneration. In collaboration with

Dr. Katherine K. Sanford and Dr. Ram Parshad (Department of Pathology, Howard University College of Medicine), cytogenetic tests which have the potential of showing very large differences in radiation-induced chromosomal aberrations between normal and Alzheimer disease (AD) cells are being developed. These studies will also hopefully help us better understand the pathogenesis of this disease. Normal and AD fibroblast strains in either the G<sub>1</sub>- or G<sub>2</sub>-phases of the cell cycle are being irradiated. Caffeine, a strong inhibitor of DNA repair during S phase, was added in some experiments. We have tested 6 AD (2 sporadic and 4 familial) and 10 normal fibroblast strains in the G<sub>1</sub> test and 7 of the AD and 10 of the normal strains in the G<sub>2</sub> test. In the G<sub>1</sub> test there were significant differences only in the presence of caffeine between the AD and normal cells in the total number of chromatid gaps and breaks per 100 metaphase cells. These G<sub>1</sub> results indicate that the normal cells repair the light-induced damage in the presence or absence of caffeine, while the AD cells cannot repair the damage in the presence of caffeine. Each normal strain could readily be distinguished from each AD strain in the G-1 test, there being no overlap in values between normal and AD strains. These G-1 results indicate that the normal cells repair the light-induced damage and that the AD cells cannot repair the damage. In the G-2 test (even in the presence of Ara-c), not every normal strain could consistently be distinguished from every AD strain. Because the G-1 and G-2 tests with fibroblasts are very difficult to perform and to evaluate cytogenetically, we have attempted to adapt the G-2 test to lymphoblastoid cell lines and to PHA-stimulated peripheral blood lymphocytes. We have so far been unsuccessful using lymphoblastoid lines, but the peripheral blood test results are promising. We plan to continue trying to perfect a useful test utilizing the latter cells.

Ex Vivo and In Vivo Manipulations of Keratinocyte Gene Expression  
(Dr. Jonathan Vogel):

The goal of this newly-established laboratory is to stably introduce and express foreign genes into keratinocytes of miniature swine (MS) epidermis. Dr. Vogel has successfully used both an *ex vivo* approach and a direct *in vivo* approach to introduce genes into miniature swine epidermis. The inserted gene in these studies was the  $\beta$ -galactosidase gene driven by strong viral promoters such as CMV, RSV, and SV40. In the first *ex vivo* approach, keratinocytes are isolated from MS epidermis; transfected with the  $\beta$ -galactosidase gene which has been coated with liposomes or cationic lipids; and grafted back onto the donor pig as keratinocyte sheets. For this *ex vivo* approach, he determined the optimal growing conditions of MS keratinocytes in tissue culture by analyzing different substrates on which to grow the cells, by testing different formulations of growth factors in culture media, and by optimizing the Ca<sup>+2</sup> concentration of the culture media. For transfection purposes, he also optimized the lipid composition of the coating liposomes and the lipid to DNA ratio. Using these conditions, he has been able to transfect approximately 50% of plated keratinocytes. The second *ex vivo* approach is to transfect DNA into epidermal keratinocytes of a skin organ culture. This has also been successful and sometimes results in gene uptake and expression around hair follicles. He has also used two different *in vivo* approaches to directly introduce genes into MS epidermal keratinocytes. The first *in vivo* approach is to inject the DNA mixture directly into a skin blister where epidermis has been separated from underlying dermis. The goal is to transfect



the rapidly proliferating basal keratinocytes which are re-epithelializing the dermal base of the blister. This approach has been successful in introducing genes into both the re-epithelializing keratinocytes as well as the keratinocytes in the epidermis of the blister roof. The second *in vivo* approach used is to inject the DNA mixture sub-epidermally (superficial dermis) with the resultant uptake and expression of our gene in keratinocytes of the overlying epidermis. To increase the number of keratinocytes containing and expressing the gene *in vivo*, he has begun developing methods to *in vivo* select for these transfected keratinocytes in a manner analogous to selection in tissue culture for stably integrated clonal cells using selecting agents such as the aminoglycoside antibiotics, G418 (Geneticin) and hygromycin B. Plasmids containing both the  $\beta$ -galactosidase gene and genes conferring resistance to the aminoglycoside antibiotics have been constructed. The plasmids which contain the resistance genes will be used to select for keratinocytes which have been transfected with them. Pilot studies have shown that topical application of the aminoglycoside antibiotics to MS epidermis is capable of killing normal keratinocytes, suggesting that *in vivo* selection is a feasible method. Additionally, this technique of topical epidermal selection can be applied and used in both the *ex vivo* and *in vivo* approaches described above.



Project DescriptionMajor Findings:

We have continued our collaboration with Drs. Katherine S. Sanford and Ram Parshad (Department of Pathology, Howard University College of Medicine) in the development of cytogenetic tests which have the potential of showing very large differences in radiation-induced chromosomal aberrations between normal and Alzheimer disease (AD) cells. We irradiate normal and AD fibroblasts in a 37-degree walk-in incubator with cool-white fluorescent light, while they are attached to glass coverslips in Leighton culture tubes or to the inner surface of plastic culture flasks. To test for repair of damage inflicted during the G-1 phase, cells were exposed to the light for 5 hours and incubated an additional 15 hours, during the last hour of which Colcemid was added. Caffeine, a strong inhibitor of DNA daughter-strand repair during S phase, was added to some of the cultures for the 15-hour incubation period. To test for repair of damage inflicted during the G-2 phase, cells were exposed for 2 hours to the light, and cells entering metaphase from 0.5-1.5 or 0.5-2.5 hours after the 2-hour light exposure were arrested by Colcemid. Beta-cytosine arabinoside (ara-C), an inhibitor of the repair replication required for completing DNA excision repair, was added to some of the cultures 10 minutes after the 2-hour light exposure. In the G-1 test, we have studied 10 normal fibroblast strains, 2 sporadic AD strains, and 4 familial AD strains (1 from one family; 3 from another). In the G-2 test we have studied 10 normal strains, 1 sporadic AD strain, and 6 familial AD strains (1 from one family; 5 from another). In the G-1 test there were no significant differences, in the absence of caffeine, between the AD and normal cells in the total number of chromatid gaps and breaks per 100 metaphase cells. However, the normal strains had no significant caffeine-induced increases in their fluorescent light-induced chromatid breaks per 100 metaphase cells, their average increase being only 0.4 while the corresponding value for the 6 AD strains was 33.3. Each normal strain could readily be distinguished from each AD strain in the G-1 test, there being no overlap in values between normal and AD strains. These G-1 results indicate that the normal cells repair the light-induced damage and that the AD cells cannot repair the damage. In the G-2 test, in the absence of ara-C, there were no significant differences between the 10 normal strains and the 6 familial AD strains. However, the 10 normal strains had ara-C-induced increases of light-induced chromatid gaps and breaks averaging 32.5 per 100 metaphase cells, while the 6 AD strains had increases averaging only 4.9. One interpretation of these G-2 test results is that the normal strains attempt to repair the light-induced damage in G-2 by making incisions at the sites of damage but are unable to fill in the resulting spaces by DNA repair replication because of the ara-C, while the AD cells do not perform the initial repair incision at sites of light-induced damage and thus create no spaces to be blocked by the ara-C. It should be noted, however, that in the G-2 test, unlike the situation in the G-1 test, not every normal strain could consistently be distinguished from every AD strain and not every AD strain could be consistently distinguished from every normal strain, because of the following results: a) one normal strain gave the AD

test-phenotype when Colcemid was present for 2 hours but gave the normal test-phenotype when Colcemid was present for only 1 hour; b) one sporadic AD and one familial AD strain gave the normal test-phenotype when Colcemid was present for 2 hours but gave the AD test-phenotype when Colcemid was present for only 1 hour. Thus, the G-2 test is not as reliable as the G-1 test in identifying individual AD strains from normal strains.

Because the G-1 and G-2 tests with fibroblasts are very difficult to perform and to evaluate cytogenetically, we have attempted to adapt the G-2 test to lymphoblastoid cell lines and to PHA-stimulated peripheral blood lymphocytes. We have so far been unsuccessful using lymphoblastoid lines, but the peripheral blood test results are promising. We plan to continue trying to perfect a useful test utilizing the latter cells.

We have completed two studies concerning the relation between clinical features of XP and the patients' DNA-repair defects. 1) We have described the neurological status of the first XP patient found to have developed XP neurological disease in adulthood. She had previously been designated as the XP complementation-group C patient XP1BE whose cultured cells are among the most widely studied of all XP patients. While she is still asymptomatic at 47 years of age, we have documented that her presymptomatic disease is progressive and qualitatively similar to, but quantitatively milder than, XP neurological disease in children. This case indicates that defective DNA repair can cause neurodegeneration in adults as well as in children. We have overcome three obstacles which had heretofore prevented recognition of this adult-onset form of XP neurological disease: a) we recognized the rarely inherited DNA-repair defect required to produce such a late-onset of neurodegeneration; b) we maintained vigorous and successful treatment of the patient's malignancies, thereby preventing her death prior to the adult age at which the neurodegeneration became identifiable; and c) we learned from our previous clinical study of XP teenagers with the late-onset type of the juvenile-onset form of XP neurological disease that sensorineural deafness and signs of a peripheral neuropathy are among the earliest manifestations of XP neurological disease. 2) We have concluded our collaboration with Drs. Wilhelm A. Bohr and Michele K. Evans in which we studied the repair of ultraviolet radiation (UV)-induced cyclobutane pyrimidine dimers in the active dihydrofolate reductase (DHFR) gene, the active *c-myc* protooncogene, and the inactive delta-globin gene in normal and XP group-A, C, D, and F fibroblast strains. In this procedure, isolated DNA from fibroblasts was restricted, separated from replicated DNA, nicked at dimers with T-4 endonuclease V, electrophoresed in alkaline agarose, and subjected to Southern hybridization. In the group-A and D strains, as well as in the F strain, less than 20% of normal repair of dimers was found in the three genes. The two group-C strains removed approximately 45-60% of the dimers from their DHFR and *c-myc* genes. The group-F strain, in comparison with the group-A, C, and D strains we studied, is known to have the highest post-UV survival and to be from the patient with the latest age of onset of skin cancer and with the least likely chance of developing XP neurologic disease. Thus, we conclude that the repair of dimers in active genes does not determine cell survival or these clinical manifestations of XP.

Publications:

Sequin LR, Ganges MB, Tarone RE, Robbins JH. Skin cancer and chromosomal aberrations induced by ultraviolet radiation: Evidence for lack of correlation in xeroderma pigmentosum variant and group E patients, *Cancer Genet Cytogenet* 1992;60:111-116.

Robbins JH, Brumback RA, Moshell AN. Clinically asymptomatic xeroderma pigmentosum neurological disease in an adult: Evidence for a neurodegeneration in later life caused by defective DNA repair, *Europ Neur* 1992;33:188-190.

Evans MK, Robbins JH, Ganges MB, Tarone RE, Nairn RS, Bohr VA. Gene-specific DNA repair in xeroderma pigmentosum complementation groups A, C, D, and F: Relation to cellular survival and clinical features. *J Biol Chem* 1993;268:4839-4847.

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

PROJECT NUMBER

Z01 CB 03657-19 D

PERIOD COVERED

October 1, 1992 to September 30, 1993

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,

P.I.: S.I. Katz, Branch Chief, Dermatology Branch, DCBDC, NCI

OTHER: M. Hertl, Guest Researcher, Dermatology Branch, DCBDC, NCI  
 A. Cavani, Guest Researcher, Dermatology Branch, DCBDC, NCI  
 E. Dugan, Medical Staff Fellow, Dermatology Branch, DCBDC, NCI  
 A. Blauvelt, Medical Staff Fellow, Dermatology Branch, DCBDC, NCI  
 C. Enk, Visiting Associate, Dermatology Branch, DCBDC, NCI

COOPERATING UNITS (if any)

Dermatology Dept., USUHS, Bethesda  
 Experimental Immunology Branch, DCBDC, NCI  
 Laboratory of Immunology, NIAID

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

5

PROFESSIONAL:

4

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

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. When murine epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells for allogeneic and autologous T cells. We have therefore utilized cultured Langerhans cells for the generation of primary immune responses in resting unsensitized T cells. We have demonstrated that when cultured cells are modified with hapten, they can generate primary immune responses. The sensitized T cells thus generated respond preferentially to the same hapten in vitro. We have utilized phenotypic and functional assays to determine whether Langerhans cells from patients infected with HIV are altered. Studies to date indicate that Langerhans cells function at least as well as monocytes in presenting autologous, allogeneic and protein antigens to T cells from HIV-infected individuals. We have also been studying the early cell and molecular events which occur after hapten painting of skin of nonsensitized mice to identify potentially important Langerhans cell and keratinocyte alterations. In response to hapten painting, activated Langerhans cells appear in vivo and there is a differential upregulation of epidermally-derived cytokine mRNAs. We have found that Langerhans cell-derived IL-1 $\beta$  is the earliest cytokine mRNA to be activated. Furthermore, we have identified that IP-10, MIP-2 and IL-10 are produced by keratinocytes. We have also demonstrated that IL-1 $\beta$  plays a critical role in the "activation" of Langerhans cells. We are currently assessing cytokine mRNAs from human skin before and after in vivo or in vitro exposure to UV radiation. Studies to date indicate that IL-10 mRNAs can regularly be detected after UV radiation of human skin.

Project DescriptionMajor Findings:

We are continuing our studies of the very earliest events which occur after skin is exposed to haptens and other chemicals, and have found that within 24 hours there is "activation" of Langerhans cells as demonstrated by their expressing greatly increased amounts of class II MHC on their surfaces, as well as their becoming much more potent antigen presenting cells than are "unstimulated" Langerhans cells. In addition we are assessing changes in epidermis-derived cytokine mRNA levels early in the afferent phase of contact sensitivity. We are using a sensitive reverse transcriptase-PCR-technique to quantitatively compare the patterns of mRNA regulation of the following: class II MHC I-A $\alpha$ , TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , interferon (IFN)- $\gamma$ , GM-CSF, IFN-induced protein 10 (IP-10) and macrophage inflammatory protein 2 (MIP-2). Enhanced LC-derived IL-1 $\beta$  mRNA signals are detected as early as 15 min after skin painting with allergens. While TNF- $\alpha$ , IFN- $\gamma$  and GM-CSF mRNAs are upregulated after application of allergens, irritant and tolerogens, class II MHC I-A $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IP-10 and MIP-2 mRNAs are upregulated only after allergen painting. Depletion of specific cell populations demonstrated that Langerhans cells are the primary source of the IL-1 $\beta$  and class II MHC I-A $\alpha$  mRNA's, keratinocytes are the primary source of the TNF- $\alpha$ , IL-1 $\alpha$ , IP-10 and MIP-2, and infiltrating T lymphocytes are the source of the IFN- $\gamma$ . The studies demonstrate that LC-derived and certain keratinocyte-derived cytokine mRNAs are selectively upregulated by allergens in the very early afferent phase of contact sensitivity. As the earliest manifestation of LC activation is the accumulation of increased amounts of IL-1 $\beta$  mRNA in LC within 15 min after exposure to contact allergens, we assessed the functional role of IL-1 $\beta$ . We found that IL-1 $\beta$  mimics the effects of allergens on LC class II MHC antigen expression, accessory cell activity and mobilization and simulates the effects of contact allergens on LC- and keratinocyte-derived cytokine mRNA signals. In aggregate, our studies show that IL-1 $\beta$  plays an essential role in the initiation of primary cutaneous responses. We have also demonstrated that IL-10 mRNA and protein production by keratinocytes is enhanced after application of contact allergens. Recently, we have shown that IL-10 inhibits the ability of Langerhans cells to present antigens to Th1 type helper T cell clones. No effect of IL-10 treatment of Langerhans cells was seen when Th2 type helper T cell clones were used.

To determine the presence of IL-10 mRNA in human keratinocytes and to study the regulation of IL-10 gene transcription by ultraviolet B radiation, we isolated mRNA from cultured human keratinocytes at different times after exposure to varying intensities of ultraviolet radiation (UVB) and used an RT-PCR technique to amplify the mRNA. Whereas non-irradiated cultured keratinocytes expressed no detectable IL-10 message, UVB radiation induced IL-10 signals 6 and 24 h post-irradiation. To determine whether UVB induces IL-10 transcription in vivo, we similarly analyzed mRNA from human epidermal cells by removing tops of suction blisters that were induced 18 h after exposure to 4 MED UVB radiation. Although IL-10 mRNA was expressed in non-UVB exposed epidermis in some individuals, it was markedly enhanced after UVB radiation. These data demonstrate that IL-10 gene expression in cultured

human keratinocytes is inducible by UVB in vitro and that keratinocyte IL-10 gene expression is also enhanced by UVB in vivo. We propose that human keratinocyte-derived IL-10 may regulate inflammatory skin reactions and be responsible for some of the immunosuppressive properties of UVB.

During this past year we have initiated studies that assess the phenotypic and functional characteristics of epidermal Langerhans cells in the skin of patients with AIDS. In our first cohort of patients, we found that Langerhans cells function at least as well as peripheral blood monocytes in the activation of T cells. We are continuing these studies and attempting to use, as responder cells, T cells from nonaffected identical twins. As well, we are assessing the ability of HIV to infect Langerhans cells from HIV-infected patients as well as normal human epidermal cells in vitro. These studies will hopefully provide insight into the role of skin as an initiator of inflammatory and perhaps neoplastic lesions in skin of HIV-infected patients. (Time devoted to AIDS is 25%)

#### Publications:

Enk A, Katz SI. Identification and induction of keratinocyte-derived IL-10, *J Immunol* 1992;149:92-95.

Cohen PJ, Katz SI. Cultured human Langerhans cells process and present intact protein antigens, *J Invest Derm* 1992;99:331-336.

Katz SI. Inherited and acquired blistering diseases (Editorial), *New Eng J Med* 1992;327:196-197.

Enk A, Katz SI. Early events in the induction phase of contact sensitivity, *J Invest Dermatol* 1992;99:39S-41S.

Gress RE, Katz SI, Lucas PJ. Human CD8<sup>+</sup> xenoreactive T cells mediate tissue injury in vitro, *Transplantation* 1993 (in press).

Kopp JB, Rooney JF, Wohlenberg C, Dorfman N, Marinos NJ, Bryant JL, Katz SI, Notkins AL, Klotman PE. Cutaneous disorders and viral gene expression in HIV-1 transgenic mice, *Aids Research and Human Retroviruses*, 1993;9:271-279.

Enk AH, Angeloni VL, Udey MC, Katz SI. An essential role for Langerhans cell-derived IL-1 $\beta$  in the initiation of primary immune responses in skin, *J Immunol* 1993;150:3698-3704.

Katz SI. The skin immune system: allergic contact dermatitis as a paradigm, *J Dermatol* (in press).

Enk AH, Angeloni VL, Udey MC, Katz SI. Inhibition of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance, *J Immunol* 1993 (in press).



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

PROJECT NUMBER

Z01 CB 03659-19 D

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Treatment and Pathogenesis of Skin Cancer and Disorders of Keratinization

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

P.I.: J.J. DiGiovanna, Expert Scientist, Dermatology Branch, DCBDC, NCI  
 Other: I. Tokar, Registered Nurse, Clinical Center Nursing  
 Maria Turner, M.D., Medical Officer, Dermatology Branch, DCBDC, NCI

COOPERATING UNITS (if any)

See next page

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The goal of these studies is to explore the efficacy, toxicity, and mechanisms of action of new treatments, and to better understand the pathogenesis of dermatologic diseases, particularly skin cancer and the disorders of keratinization. During the last decade over 300 patients studied have characterized the efficacy and toxicity of isotretinoin and etretinate in the treatment of a variety of disorders. Patients requiring long-term retinoid therapy are monitored to characterize chronic skeletal toxicities. The high rate of peripheral skeletal involvement after chronic etretinate therapy was first identified in these patients. A trial of Peptide T for psoriasis, a synthetic oligopeptide which has been associated with improvement in HIV related psoriasiform eruptions, showed minimal efficacy. We demonstrated the effectiveness of oral isotretinoin as a chemopreventive agent in patients with high rates of skin cancer formation. They are now maintained on long-term isotretinoin for cancer prevention. A study of recombinant human interferon gamma for basal cell carcinoma is nearing completion. All tumors treated have become smaller, but few underwent complete histological regression. In collaboration, we have identified linkage of the nevoid basal cell carcinoma syndrome gene to 9q31. In tumors, loss of heterozygosity in that region suggests that a mutation in a tumor suppressor gene is the probable cause of the disease. Linkage analysis is being pursued for fine mapping of the region. We were the first to identify linkage of the epidermolytic hyperkeratosis gene to the type II keratin gene cluster on chromosome 12q. We have now identified mutations in 11 families and have identified 7 distinct clinical phenotypes. The correlation between these mutations and clinical phenotypes should lead to a better understanding of the relationship between keratin structure and function.

Cooperating Units

K. Kraemer, Senior Investigator, Lab. Molecular Carcinogenesis  
Sherri J. Bale, Ph.D., Acting Chief, Genetics Studies Sect., LSB, NIAMS  
John Compton, Ph.D., Sr. Staff Fellow, Lab. Skin Biology, NIAMS  
Peter Steinert, Ph.D., Chief, Lab. Skin Biology, NIAMS  
Allen E. Bale, M.D., Dept. of Genetics, Yale Univ. School of Medicine  
Nicholas Patronas, Radiology Department, Clinical Center  
James Reynolds, M.D., Chief, Clinical Studies Sect., Nuclear Med., CC

Major Findings:

The efficacy and toxicity of isotretinoin as a chemopreventive agent is being further studied in a series of 9 patients with xeroderma pigmentosum or the nevoid basal cell carcinoma syndrome. Additional patients are being screened for inclusion into this study. Initial results identified an improvement of great magnitude in the rate of new skin cancer formation while on high dose (2.0 mg/kg/day) isotretinoin therapy. After a 2 year period, isotretinoin was discontinued to determine if benefit would persist. Skin cancers began to occur at the pretreatment rate within 2 to 3 months. This suggests that isotretinoin's chemopreventive action is occurring at a late stage of carcinogenesis. The beneficial effect was highly statistically significant. All patients had mucocutaneous side effects, many had laboratory abnormalities and two had skeletal toxicity. In a further study, patients were then restarted on isotretinoin at a low dose (0.5 mg/kg/day) in an effort to minimize toxicity. One patient had similar benefit on both the low and high dose treatments. Four patients had less improvement on low compared to high dose, suggesting a dose-response. Patients with inadequate response to low dose are being treated at intermediate doses (1.0 - 1.5 mg/kg/day) in an effort to achieve adequate chemoprevention with minimal toxicity.

Patients with a variety of ichthyoses, Darier's disease, pityriasis rubra pilaris, and related conditions have maintained clinical improvement for more than a decade while being treated with the isotretinoin or etretinate. Most of these conditions have no effective alternative therapy. Chronic retinoid bone toxicity has been extensively studied in these patients. This toxicity is similar to the disorder DISH (diffuse idiopathic skeletal hyperostosis). Our group was the first to identify the high frequency of involvement of peripheral skeletal tissue involvement in etretinate treated patients. This peripheral skeletal toxicity also occurs with isotretinoin. Monitoring of these patients will enable us to further define the parameters of these toxicities.

Osteoporosis has been observed in chronic hypervitaminosis A. Our unique ability for long term follow-up of these patients allows us to assess this potential toxicity in patients chronically treated with isotretinoin or etretinate. A study is underway in collaboration with James Reynolds from the Nuclear Medicine Department. To date, dual photon bone densitometry has been performed on 29 of these patients to assess bone density.

Six patients with psoriasis were treated for eight weeks with intranasal Peptide T. Peptide T, which interacts with the CD4 receptor, is being studied in NIMH for the treatment of HIV associated dementia. The baseline neuropsychiatric and pharmacokinetic data obtained in these studies on a non-HIV infected population are being utilized to characterize the drug's actions in HIV patients. Only minimal benefit was observed using either the intranasal preparation and in a subsequent topical study. Time devoted to these AIDS-related activities is 5%.

Five patients have each had one basal cell carcinoma treated with 0.1 mg/M<sup>2</sup> (body surface area) intralesional recombinant gamma interferon. All tumors decreased in size during treatment and one tumor was identified to have undergone complete histological regression at post treatment excision. Some treated areas developed clinical milia, suggesting that the lesions were being induced to differentiate. Histologic examination showed that keratinization was being induced as manifested by the development of pseudohorn cysts and dermal aggregates of keratin. The second phase of the study is ongoing, using a higher dose of gamma interferon to try to achieve greater efficacy. A sixth lesion was treated at 0.5 mg/M<sup>2</sup> and underwent complete histological regression but with moderate inflammation. The study is nearing completion with additional lesions being treated at 0.25mg/M<sup>2</sup>.

As part of our study of genodermatoses, we have identified and characterized a novel, cystic bone abnormality in patients with Darier's disease. Ten of 17 patients surveyed had cystic bone lesions, one had a history of fractures. Bone cysts were identified both in patients on retinoid therapy and also in those who had never been treated with retinoids. Bone scans on 2 patients did not show increased uptake of radionuclide. The abnormality in Darier's disease was previously thought to be limited to the skin and mucous membranes. This work has identified that the abnormality in this disorder is not confined to the integument but can involve other organ systems.

We have formed collaborative groups to study the clinical spectrum and map the genes for a series of genodermatoses. Our collaboration, started in 1985, with Allen Bale and more recently with the Lab. of Skin Biology, to map the gene for the nevoid basal cell carcinoma syndrome (NBCC). We have identified tight linkage between the NBCC gene and polymorphic markers in 9q31. Consistent allelic loss of this region in the tumors from sporadic basal cell carcinomas and hereditary tumors suggests that the cause of the tumors in this condition may be the loss of function of a tumor suppressor gene. It is now possible to provide prenatal diagnosis in informative families. Linkage analysis is being pursued for fine mapping of this region.

In our study with the Lab. of Skin Biology designed to better clinically characterize and to map the genes for the disorders of keratinization we have focused on epidermolytic hyperkeratosis (EHK) and the recessive ichthyoses. We traveled to the midwestern U.S. to examine and obtain tissue samples from one family with 20 affected members in 3 generations. Linkage analysis in

this family demonstrated that the gene for EHK was on chromosome 12q in the vicinity of a cluster of keratin genes. There was no recombination with the keratin 1 locus. Sequencing of the gene in affecteds and a large series of controls showed a leucine to proline change which cosegregated with the disease. This data, supported by a structure-function relationship (impaired ability of a peptide fragment with the mutation to interfere with keratin aggregation) provides extremely strong evidence that this mutation causes EHK in this family. To date we have examined 53 EHK patients and their unaffected relatives from 21 families. We have identified striking heterogeneity in clinical presentation between families with EHK. We have been able to distinguish 7 clinical phenotypes on the basis of palm/sole hyperkeratosis, character and extent of scale, and presence of erythroderma. To date, mutations have been identified in 11 families. Those families with hyperkeratosis of the palms and soles have had mutations in keratin 1. Those without hyperkeratosis of the palms and soles have had mutations in keratin 10. Further correlation of these clinical phenotypes and mutations should lead to a better understanding of the relationship between intermediate filament structure and function, and the maintenance of epidermal integrity.

Because there is more information from consanguineous families with affected children, and because consanguinity is more common in Egypt we initiated a collaboration with Dr. Nemat Hashem, Ain Shams University, Cairo, Egypt. Under the auspices of the Office of International Health, we traveled to Cairo to examine patients and obtain specimens on 48 individuals from 16 families. We are working to establish a collaboration with investigators in Israel, another area where consanguinity is more common.

We have also established a collaboration with the Istituto Dermopatico Dell' Immacolata, Rome, Italy, to study families with ichthyosis vulgaris, Hailey-Hailey and Darier's disease.

#### Publications:

Compton JG<sup>1</sup>, DiGiovanna JJ<sup>1</sup>, Santucci SK, Kearns KS, Amos CI, Abangan DL, Korge BP, McBride WO, Steinert PM, Bale SJ. Linkage of epidermolytic hyperkeratosis to the type II keratin gene cluster on chromosome 12q, Nature Genetics 1992;1:301-305.

<sup>1</sup> JGC and JJD contributed equally to this project.

Chipev CC, Korge BP, Markova N, Bale SJ, DiGiovanna JJ, Compton JG, Steinert PS. A leucine to proline mutation in the H1 domain of keratin 1 causes epidermolytic hyperkeratosis, Cell 1992;70:821-828.

DiGiovanna JJ. Retinoids for the future: Oncology, J Am Acad Derm 1992;27:(6) Pt 2:S34-S37.

Kraemer KH, DiGiovanna JJ, Peck GL. Chemoprevention of skin cancer in xeroderma pigmentosum, J Derm 1992;19:715-718.

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DiGiovanna JJ. Diversity of melanoma in humans, *Clin Chem Enzym Comm* (in press).

Lippman SM, Ankrum M, DiGiovanna JJ. Oral retinoids in skin cancer, *Cancer Bull* (in press).

DiGiovanna JJ, Kraemer KH, Peck GL, Abangan DL. Oral isotretinoin is effective in the prevention of skin cancer in patients with xeroderma pigmentosum. In: Pennington Center Nutrition Series, vol 3, Bray GA and Ryan DH, eds. *Vitamins and cancer prevention*. Louisiana State University Press, 1993;188-195.

Peck GL, DiGiovanna JJ, Kraemer KH, Tangrea JA, Gross EG. Treatment and prevention of basal cell carcinoma with oral isotretinoin. In: DePalo G, Sporn M, Veronesi U, (Eds). *Progress and perspectives in chemoprevention of cancer*, Raven Press, New York, 1992;149-159.

DiGiovanna JJ. Prevention of cancer of the skin due to sun exposure. In: DeVita VT, Hellman S, Rosenberg SA, eds. *Philadelphia: J.B. Lippincott Co.*, 1991;8:1-12.

Peck GL, DiGiovanna JJ, Kraemer KH, Tangrea JA, Gross EG. Treatment and prevention of basal cell carcinoma with oral isotretinoin. In Roenigk RK and Roenigk HH (Eds), *Surgical Dermatology*, Martin Dunitz, London, 1993;155-163.

Peck GL, DiGiovanna JJ. Retinoids. In: Fitzpatrick TB, et al eds. *Dermatology in general medicine*. New York: McGraw-Hill, 1992 (in press).

Lippman SM and DiGiovanna JJ: Retinoids in skin cancer. In Hong WK, Lotan R (Eds), *Retinoids in Oncology* (in press).

Bale SJ, Compton JG and DiGiovanna JJ. Epidermolytic hyperkeratosis. In Goldsmith L (Ed), *Molecular Biology of the Skin*, (in press).

Peck GL and DiGiovanna JJ. Synthetic retinoids in dermatology, In, Sporn MB, Roberts AB and Goodman DS, *The Retinoids*, Academic Press, Orlando (in press).

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

PROJECT NUMBER

Z01 CB 03667-09 D

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Characterization of Epidermal Cell Adhesion Molecules with Autoantibodies

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, DCBDC, NCI  
OTHER: Sarolta Karpati, M.D., Visiting Scientist, Dermatology Br., DCBDC, NCI  
Joo-Young Roh, M.D., Ph.D., Visiting Scientist, Dermatology Br.,  
DCBDC, NCI  
Todd Plott, M.D., Medical Staff Fellow, Dermatology Br., DCBDC, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF 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 nomenclature 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. Specifically, we have found that autoantibodies from these patients, who develop blistering diseases due to defects in epidermal cell adhesion, are directed against adhesion molecules. We are characterizing, by immunochemical and molecular biologic means, the antigens defined by three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF). This then allows us to study their cell biologic function. BP antigen is a component of the hemidesmosome, a basal-cell substrate adhesion junction. Using a polymerase chain reaction technique called rapid amplification of cDNA ends, we have finished cloning cDNA with the full length coding sequence for this molecule. Analysis of its deduced amino acid sequence indicates that BP antigen is a totally intracellular protein that has marked amino acid and structural homology with desmoplakin I, a desmosome plaque protein, and with plectin, a keratin-associated protein. We have also cloned cDNA with the full length coding sequence for PV antigen. The deduced amino acid sequence of this antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules and is closely related to the PF antigen. PV patients have antibodies against the amino-terminal domain of this molecule, an area thought to be important in its adhesion function, and these antibodies can cause loss of adhesion of epidermal cells in an animal model of disease. In order to study the biological function of PV antigen, we have transfected eukaryotic cells with cDNA that encodes its extracellular domains as well as mutated cDNA that encodes a truncated extracellular domain and a normal cytoplasmic domain.

## Project Description

### Major Findings:

Overlapping cDNAs encoding the entire 230-kD bullous pemphigoid (BP) antigen have been cloned by screening cDNA libraries and by using the polymerase chain reaction rapid amplification of cDNA ends (RACE) technique.

Sequence analysis indicates that BP antigen is similar in structure, charge periodicity, and amino acid sequence to desmoplakin I, a desmosomal plaque protein, and to plectin, a keratin-binding protein. There is no transmembrane sequence.

cDNA cloning of pemphigus vulgaris antigen (PVA) indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules. PVA is most closely related to desmoglein I, which is also in the cadherin supergene family, and is pemphigus foliaceus antigen. PVA and desmoglein I have very similar structures. Each have repeating, homologous extracellular domains, a transmembrane segment, and similar cytoplasmic domains.

Pemphigus vulgaris patients' sera have antibodies that bind the amino-terminal extracellular domain of PVA, a region of cadherins thought to be important for their function of homophilic binding. IgG from these patients' sera, affinity purified on this domain, is capable of causing the pathology of pemphigus vulgaris blisters (i.e. loss of epidermal cell adhesion) in a well-established neonatal mouse model of disease. In this model, these affinity purified antibodies localize to the core of separating desmosomes (cell-cell adhesion junctions), as determined by immunogold electron microscopy.

Rabbit antibodies against the extracellular domains of PVA have been raised and shown to be specific by immunofluorescence, immunoblotting, and immunoprecipitation.

These rabbit antibodies and the antibodies affinity purified from patient sera on the extracellular domains of PVA have been used to ultrastructurally localize PVA by immunogold electron microscopy. These studies show that PVA is found in desmosomes, the same location as desmoglein I. Therefore, PVA is homologous to desmoglein I at the level of amino acid sequence, protein structure, and ultrastructural localization.

A chimeric cDNA that encodes the extracellular domains of PVA and the transmembrane and cytoplasmic domains of E-cadherin has been constructed and used for transient and permanent transfections of eukaryotic cells. Immunoblotting, immunofluorescence, and immunoprecipitation studies of these cells indicates that the cDNA is expressed as a transmembrane protein whose cytoplasmic domain binds catenins, just as the cytoplasmic domain of whole E-cadherin. These transfected cells will be used to further study the biological function of PVA.

Publications:

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Rappersberger K, Roos N, Stanley JR. Immunomorphologic and biochemical identification of the pemphigus foliaceus autoantigen within desmosomes, *J Invest Dermatol* 1992;99:323-330.

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Tang A, Amagai M, Granger LG, Stanley JR, Udey MC. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin, *Nature* 1993;361:83-85.

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Hashimoto T, Ebihara T, Ishiko A, Shimizu H, Bhogal BS, Black MM, Stanley JR, Nishikawa T. Comparative study of bullous pemphigoid antigens among Japanese, British, and U.S. patients indicates similar antigen profiles with the 170-kD antigen present both in the basement membrane and on the keratinocytes cell membrane, *J Invest Dermatol* (in press).

Elgart GW, Stanley JR. Cloning of the 5' mRNA for the 230-kD bullous pemphigoid antigen by rapid amplification of cDNA ends, *J Invest Dermatol* (in press).

Patient Application: pemphigus vulgaris antigen cDNA



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

PROJECT NUMBER

Z01 CB 03669-04 D

PERIOD COVERED

October 1, 1992-September 30, 1993

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

Regulation of Cutaneous Accessory Cell Activity in Health and Disease

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

P.I.: Mark C. Udey, M.D., Ph.D., Expert, Dermatology Branch, DCBDC, NCI

Other: Teresa A. Borkowski, M.D., Medical Staff Fellow, DB, DCBDC, NCI  
 Min-Geol Lee, M.D., Ph.D., Visiting Associate, DB, DCBDC, NCI  
 Kathryn Schwarzenberger, M.D., Medical Staff Fellow, DB, DCBDC, NCI  
 Byron J. Van Dyke, HHMI-NIH Research Scholar

COOPERATING UNITS (if any)

Alfred Singer, M.D., Chief, Experimental Immunology Branch, DCBDC, NCI; Susan O. Sharrow, Senior Investigator, EIB, DCBDC, NCI; Andrew Farr, Ph.D., Associate Professor, Department of Structural Biology, University of Washington, Seattle, WA

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

5.33

PROFESSIONAL:

4.33

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 unrounded type. Do not exceed the space provided.)

The goal of the laboratory is to understand the cell biology of epidermal Langerhans cells (LC). We have completed our studies of expression of the costimulatory molecule B7 by epidermal cell (EC) subpopulations. Using primers specific for murine B7 and quantitative RT-PCR, we determined that B7 mRNA was 100-1000 fold more abundant in 72 h cultured BALB/c EC (LC and keratinocytes) than in fresh EC. Cell depletion studies carried out with monoclonal antibodies and magnetic beads demonstrated that the B7 mRNA present in cultured EC was contained in LC. Using identical conditions, essentially no B7 mRNA was identified in freshly prepared EC (LC and keratinocytes), normal or transformed keratinocytes, or fibroblasts. Two color flow cytometry, carried out with the B7-binding fusion protein CTLA4Ig, confirmed that cultured LC express abundant surface B7 while fresh LC and keratinocytes do not. These data suggest that among EC, LC are uniquely able to synthesize and express B7. In addition, levels of B7 expressed by LC are proportional to mRNA levels. More recently, we discovered that LC express E-cadherin (E-cad), one of a supergene family of calcium-dependent homophilic adhesion molecules. We also demonstrated that LC contain E-cad mRNA and that E-cad mediates adhesion of LC to keratinocytes. E-cad is not expressed by splenic or gut-associated lymph node (LN) dendritic cells (DC), but may be expressed at low levels by subpopulations of thymic DC and DC prepared from skin-associated LN. In addition, E-cad is expressed by DC propagated from blood and bone marrow. These results suggest that E-cad is a DC differentiation antigen expressed by LC, cells that may be derived from LC (skin-associated LN DC), cells that may give rise to LC (blood and bone marrow DC), and cells that may be closely related to LC (thymic DC). We have begun to use blood and bone marrow-derived DC as a model to study the biochemistry and cell biology of leukocyte E-cad. We have demonstrated that DC contain mRNA identical to that encoding E-cad expressed by nonleukocytes. E-cad immunoprecipitated from DC also comigrates with that derived from fibroblasts transfected with E-cad cDNA. We are trying to identify factors that regulate DC E-cad expression and activity and will use E-cad to study LC and DC ontogeny. We have also detected E-cad on the surfaces of resident murine epidermal  $\gamma\delta$  T cells and fetal thymocytes, suggesting that E-cad may play a more general role in leukocyte-epithelial interactions. Additional experiments will be required to determine whether or not E-cad is involved in adhesion of  $\gamma\delta$  T cells to keratinocytes, and to determine if E-cad plays a role in thymocyte development.

## Project Description

### Major Findings

We are interested in trying to better understand the cell biology of murine epidermal Langerhans cells (LC) by studying cell surface adhesion molecules responsible for interactions between LC and other cells. Initially, we studied adhesion molecules involved in interactions between LC and T cells. Studies of effects of ultraviolet radiation on expression of the intercellular adhesion molecule ICAM-1 (CD54) by LC have been completed and were detailed in the previous annual report. More recently we examined LC for evidence of expression of the B cell activation antigen B7 (BB1).

Recent studies suggest that B7, a member of the immunoglobulin supergene family, may be the single most important of the Th1 cell costimulatory molecules expressed by accessory cells. Indeed, B7-based therapy has already been successfully employed in the treatment of experimental allograft rejection and cancer. We reasoned that cultured LC might be potent accessory cells in part because they expressed B7, and that fresh LC might be less competent accessory cells because they did not express B7. Because serologic reagents were not available to us at the time we began our experiments, we designed primers based on the published nucleotide sequence for murine B7 cDNA and quantitated B7 mRNA levels using RT-PCR. B7 mRNA levels in cultured epidermal cells (LC and keratinocytes (KC)) were 100 to 1000-fold higher than those in fresh EC. In addition, B7 mRNA-derived signals were not obtained from transformed KC or fibroblasts. Depletion studies performed with appropriate monoclonal antibodies and magnetic beads indicated that essentially all of the B7 mRNA in cultured EC was contained in I-A-positive and CD45-positive LC. Using the B7-binding fusion protein CTLA4Ig (ultimately made available to us by Dr. Peter Linsley of Bristol Meyers-Squibb), we verified that cultured LC express levels of B7 at least 100-fold greater than those expressed by fresh LC or KC. These results suggest that in murine epidermis, only LC have the capacity to synthesize and express the potent costimulatory molecule B7. This may explain why LC facilitate antigen-dependent T cell activation, whereas class II MHC antigen-bearing KC (which presumably do not express B7) induce antigen-dependent unresponsiveness. Modulation of LC B7 expression by ultraviolet radiation may also convert LC from cells capable of activating T cells into cells that promote clonal anergy.

In the past year most of the effort in my laboratory has been directed towards the study of cadherins expressed by leukocytes. Cadherins comprise a growing supergene family of calcium-dependent homophilic adhesion molecules known to be involved in embryogenesis and in the maintenance of structural integrity in epithelia and the nervous system. Cadherins had not been identified on leukocytes at the time we began our studies. We determined that fresh murine BALB/c LC and KC express similar levels of E-cadherin, a cadherin initially described in epithelia. LC appear to actively synthesize E-cadherin, because LC contain E-cadherin mRNA. E-cadherin mediates adhesion of LC to E-cadherin-transfected fibroblasts and KC *in vitro*, and adhesion of LC to KC can be selectively inhibited by anti-E-cadherin monoclonal antibodies. Cultured LC, cells that may represent the *in vitro* equivalent of LC that have migrated from epidermis to regional lymph nodes, express lower levels of E-cadherin and adhere less avidly to KC.

We subsequently surveyed dendritic cells (DC) from various lymphoid tissues for E-cadherin expression. Splenic DC and DC prepared from gut-associated lymph nodes (LN) do not express E-cadherin. Results of two color flow cytometry studies suggest that skin-associated LN DC uniformly express low levels of E-cadherin, however. These latter cells may correspond to LC that have migrated from epidermis to regional LN after epicutaneous exposure to antigen. A subpopulation of thymic DC may also express low levels of E-cadherin. Methods for culturing DC from blood and bone marrow have recently been described. We have propagated DC from the blood of cyclophosphamide-pretreated mice and from the bone marrow of normal animals. Blood and bone marrow DC express various DC surface antigens, and also transiently express E-cadherin *in vitro*. We propose that E-cadherin is a differentiation antigen expressed by LC, cells that may be derived from LC

(skin-associated LN DC), cells that may give rise to LC (blood and bone marrow DC) and cells that may be related to LC (thymic DC). It also seems likely that E-cadherin plays an important role in the localization of LC in epidermis, but this has not been formally tested.

We plan to use blood and bone marrow DC as model systems to study leukocyte cadherin biochemistry and cell biology. We have already confirmed that blood DC contain full length E-cadherin mRNA transcripts, and have demonstrated that E-cadherin immunoprecipitated from surface labeled DC comigrates with that from L cells transfected with murine E-cadherin cDNA. Preliminary experiments also suggest that DC E-cadherin is noncovalently associated with catenins, intracellular proteins that have been reported to link cadherins to the cytoskeleton in nonleukocytes. In an effort to better understand how E-cadherin influences LC biology, we will attempt to identify cytokines or other factors that regulate levels of expression or binding activity of E-cadherin expressed by cultured DC. We will also seek to determine if E-cadherin expressing cultured DC can give rise to cells resembling LC in vitro (in DC-KC coculture experiments) or in vivo (after adoptive transfer).

To determine if E-cadherin expression is a general feature of murine dendritic epidermal leukocytes, we studied C3H/HeN LC and epidermal  $\gamma\delta$  T cells in parallel. Like LC, epidermal  $\gamma\delta$  T cells all express E-cadherin. Similar levels of E-cadherin were expressed by KC, LC and epidermal  $\gamma\delta$  T cells. Like E-cadherin expressed by LC and KC, E-cadherin expressed by epidermal  $\gamma\delta$  T cells is resistant to degradation by trypsin in the presence of calcium, and sensitive to trypsin proteolysis in the absence of calcium. Experiments designed to determine if E-cadherin can mediate adhesion of epidermal  $\gamma\delta$  T cells to KC have been inconclusive to date, perhaps for technical reasons.

Murine epidermal  $\gamma\delta$  T cells uniformly express V $\gamma$ 3 T cell receptors, and may be direct descendents of the V $\gamma$ 3 positive thymocytes that develop in a single wave in fetal thymus. In collaboration with Drs. Singer, Sharrow, Farr and coworkers, we have begun to study cadherin expression in developing murine thymus. Preliminary results indicate that E-cadherin is prominently expressed by fetal thymocytes. Expression is highest on day 14 fetal thymocytes and decreases with increasing gestational age. Only a minor population of thymocytes expresses E-cadherin at birth, and very few adult thymocytes express E-cadherin. Although E-cadherin expression is not restricted to CD4-negative CD8-negative cells, double negative thymocytes express the highest levels of at each gestational age. Cadherin expression by thymic epithelial cell lines is also heterogeneous. Several cell lines express E- and P-cadherin (the latter initially described in placenta); others do not express either cadherin. We will utilize these cell lines to determine if E-cadherin is involved in adhesion between prothymocytes and thymic epithelial cells in developing thymus. In addition, we will study effects of anti-E-cadherin antibodies on thymocyte development in fetal thymic explants.

Data accumulated to date is compatible with the hypothesis that E-cadherin mediates stable interactions between relatively immature lymphoid cells (LC, epidermal  $\gamma\delta$  T cells and prothymocytes) and epithelia (epidermis and thymic epithelia). In future experiments, it will be of interest to determine if cadherins are involved in other leukocyte-epithelial interactions in normal tissues and in disease.

Publications

Journal Articles:

Tang A and Udey MC. Effects of ultraviolet radiation on murine epidermal Langerhans cells: Doses of ultraviolet radiation which modulate ICAM-1 (CD 54) expression and inhibit Langerhans cell function cause delayed cytotoxicity in vitro. *J. Invest. Dermatol.* 99: 83-89, 1992.

Tang A, Amagai M, Granger LC, Stanley JR and Udey MC. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature* 361: 82-85, 1993.

Enk AH, Angeloni VL, Udey MC and Katz SI. An essential role for Langerhans cell-derived IL-1 $\beta$  in the initiation of primary immune responses in skin. *J. Immunol.* 150: 3698-3704, 1993.

Enk AH, Angeloni VL, Udey MC and Katz SI. Inhibition of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. *J. Immunol.* (In press).

Journal Supplements:

Tang A and Udey MC. Doses of ultraviolet radiation that modulate accessory cell activity and ICAM-1 expression are ultimately cytotoxic for murine epidermal Langerhans cells. *J. Invest. Dermatol.* 99: 71s-73s, 1992.

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

PROJECT NUMBER

Z01 CB 03670-01 D

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Ex vivo and in vivo manipulations of keratinocyte gene expression

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

P.I.: Jonathan Vogel, M.D., Expert, Dermatology Branch, DCBDC, NCI

Other: Edward Chan, HHMI, NIH Research Scholar

Ulrich Hengge, M.D., Visiting Fellow, Dermatology Br., DCBDC, NCI

Ruth Ann Foster, General Fellowship Trainee, Dermatology Br., DCBDC, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.08

PROFESSIONAL:

3.08

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

The goal of this laboratory is to stably introduce and express foreign genes in keratinocytes of miniature swine (MS) epidermis. We have successfully used both an *ex vivo* approach and a direct *in vivo* approach to introduce genes into miniature swine epidermis. The inserted gene in these studies was the  $\beta$ -galactosidase gene driven by strong viral promoters such as CMV, RSV, and SV40. In the first *ex vivo* approach, keratinocytes are isolated from MS epidermis; transfected with the  $\beta$ -galactosidase gene which has been coated with liposomes or cationic lipids; and grafted back onto the donor pig as keratinocyte sheets. For this *ex vivo* approach, we have determined the optimal growing conditions of MS keratinocytes in tissue culture by analyzing different substrates on which to grow the cells, by testing different formulations of growth factors in culture media, and by optimizing the  $Ca^{+2}$  concentration of the culture media. For transfection purposes, we have also optimized the lipid composition of the coating liposomes and the lipid to DNA ratio. Using these conditions, we have been able to transfect approximately 50% of plated keratinocytes. The second *ex vivo* approach is to transfect DNA into epidermal keratinocytes of a skin organ culture. This has also been successful and sometimes results in gene uptake and expression around hair follicles. We have re-grafted one of these organ cultures back onto the donor pig to see if expression of the transfected gene persists. We also have used two different *in vivo* approaches to directly introduce genes into MS epidermal keratinocytes. The first *in vivo* approach is to inject the DNA mixture directly into a skin blister where epidermis has been separated from underlying dermis. The goal is to transfect the rapidly proliferating basal keratinocytes which are re-epithelializing the dermal base of the blister. This approach has been successful in introducing genes into both the re-epithelializing keratinocytes as well as the keratinocytes in the epidermis of the blister roof. The second *in vivo* approach we have used is to inject the DNA mixture sub-epidermally (superficial dermis) with the resultant uptake and expression of our gene in keratinocytes of the overlying epidermis. To increase the number of keratinocytes containing and expressing our gene *in vivo*, we have begun developing methods to *in vivo* select for these transfected keratinocytes in a manner analogous to selection in tissue culture for stably integrated clonal cells using selecting agents such as the aminoglycoside antibiotics, G418(Geneticin) and hygromycin B. We have constructed plasmids containing both the  $\beta$ -galactosidase gene and genes conferring resistance to the aminoglycoside antibiotics. The plasmids which contain the resistance genes will be used to select for keratinocytes which have been transfected with them. Pilot studies have shown that topical application of the aminoglycoside antibiotics to MS epidermis is capable killing normal keratinocytes, suggesting that *in vivo* selection is a feasible method. Additionally, this technique of topical epidermal selection can be applied and used in both the *ex vivo* and *in vivo* approaches described above.

## Project Description

### Major Findings and Proposed Studies

The main focus of this laboratory is to develop novel methods for the stable introduction and expression of genes in keratinocytes, which constitute more than 90% of mammalian epidermis. These techniques would allow the development of a model system for *in vivo* gene therapy using keratinocytes as a vehicle. Epidermal keratinocytes containing these introduced genes could express protein products such as cytokines, growth factors, or enzymes for systemic delivery or they could express normal keratinocyte proteins which could treat specific skin diseases due to a genetic defect of an endogenous keratinocyte protein. Another use of this gene delivery system would be to experimentally elucidate the effects of introduced genes on normal keratinocyte functions such as differentiation or the ability to form normal epidermis. Additional uses would be to study how the protein products of introduced genes cross the dermal-epidermal (DE) junction and to determine what the requirements are for passage across the DE junction. Finally, introduced genes could be used as markers to assess the kinetics of stem cell populations.

Why use keratinocytes for these purposes? In addition to their important structural role of producing intermediate filaments such as the keratins, keratinocytes are also potent secretory cells and produce a broad family of cytokines and growth factors. Keratinocytes and epidermis are also easily accessible for different technical manipulations, such as keratinocyte isolation and skin grafting, and following gene therapy, the epidermis is easily assessed for the presence and/or expression of the introduced gene. Also, because it is easily accessible, additional manipulations following gene therapy can be performed, such as selecting for keratinocytes containing the introduced gene. The ability to select or enrich for stably transfected keratinocytes in an *in vivo* setting is a very important and novel aspect of our use of keratinocytes for gene therapy. In a manner analogous to selection in tissue culture where reagents such as G418 (geneticin) and hygromycin B are used to select for stably transfected clones which contain the appropriate resistance gene, we will include resistance genes on our DNA plasmid constructs and select for stably transfected keratinocytes *in vivo* using long-term topical application of the selecting agent (such as G418 or Hygromycin). This long-term *in vivo* topical selection should select for stem cells or early precursors that have stably integrated DNA. It also obviates the need for extremely high initial transfection frequencies when first introducing the genes into keratinocytes. Preliminary experiments using selectable reagents will be described below.

What are the major technical hurdles in using keratinocytes as a vehicle for gene therapy? As for all epithelia, the epidermis is a renewable tissue and if the introduced genes are not present in stem cells or very early precursors, they will be rapidly lost from the epidermis as it proliferates and differentiates. The epidermis is also not vascularized, and if systemic effects of a keratinocyte produced protein are desired, it will have to cross the DE junction to enter the vascular system. And the requirements or criteria that determine which proteins cross the DE junction have not been defined. Finally keratinocytes are difficult to manipulate in tissue culture because of their propensity to differentiate and undergo senescence, especially at higher calcium concentrations.

The miniature swine (MS) is the animal model we chose for our studies because its epidermis is structurally similar to human epidermis and correspondingly, it has been extensively used as a model in the past for wound healing and skin graft transplantation procedures in humans. We have utilized both *ex vivo* and *in vivo* approaches for introducing genes into the MS epidermis. In the *ex vivo* approaches, the genes are targeted and inserted into keratinocytes growing in tissue culture or are targeted to an intact organ culture of MS skin which contains both epidermis and underlying dermis. Following introduction of the gene into the tissue culture keratinocytes or into the keratinocytes of the organ culture epidermis, the keratinocyte sheets or organ culture grafts would be engrafted back onto the donor pig. The *in vivo* approach involves direct insertion of the gene into the keratinocytes of the MS epidermis. This approach bypasses the *ex vivo* manipulations described above, but does require that for persistent long term expression, the introduced gene needs to be targeted to either keratinocyte stem cells or early keratinocyte precursors in the proliferating basal layer of the epidermis.

The gene constructs we first introduced into keratinocytes and MS epidermis were plasmid constructs containing the indicator gene  $\beta$ -galactosidase driven by a variety of different viral promoters including CMV, RSV, and SV40 early promoters. This indicator gene was chosen because it allows assessment of gene expression on a cellular level by histochemical techniques as well as quantitative analysis of expression in a whole tissue by photometric techniques. The different viral promoters were chosen because they are known to express at high levels in a variety of different tissues and presumably would be able to express in the epidermal keratinocytes at different stages of differentiation. All the data and results presented are derived from these gene constructs. More recently, we now have constructed plasmids that contain both the  $\beta$ -galactosidase gene as well the genes which confer resistance to the aminoglycoside antibiotics G418 (Geneticin) and Hygromycin B (the aminoglycoside phosphotransferase (APH) gene and the hygromycin B phosphotransferase (hyg) gene respectively). Keratinocytes transfected with these plasmids can be selected because these plasmids contain resistance genes. Additionally, we have constructed plasmids which not only contain the  $\beta$ -galactosidase and selectable resistance genes, but also contain viral genes which maintain the plasmids in an episomal state and allow episomal replication. The advantage of these episomal plasmids is that they do not require stable integration into the chromosomes for long-term maintenance inside a cell. Regular plasmids do require stable integration (a relatively rare event following cellular uptake of DNA) for persistence, otherwise they will eventually be lost from the cell following transient transfection. It is our hope that keratinocytes transfected with these episomal plasmids will harbor and express them in an stable episomal state over a long period of time.

Since almost no information existed regarding the proper techniques to culture MS keratinocytes, our first task in pursuing the *ex vivo* approach was to develop the proper culture conditions to facilitate the rapid proliferation of keratinocytes in an undifferentiated state so they could be easily grown and repeatedly passaged. Attempts to culture MS keratinocytes using conditions developed for human keratinocytes have not been successful. We succeeded in culturing MS keratinocytes by trying a variety of different substrates, optimizing the  $\text{Ca}^{+2}$  concentration to maintain an undifferentiated morphology, and analyzing different growth factors and media. Our oldest primary MS keratinocyte culture has been carried for over 4 months and passaged over 20 times. Our future goals are to assess additional growth factors in this system, with special attention paid to those factors (SCF, LIF, GM-CSF) which can promote the growth of early precursors or stem cells, since these are the preferred cells to target for gene therapy.

In order to efficiently introduce the DNA constructs into keratinocytes, we have initially used non-retroviral methods such as liposomes or cationic lipid coatings of DNA to promote cellular uptake. By optimizing both the type of cationic lipid used and optimizing the lipid to DNA ratio, we have been able to achieve transient transfection frequencies of approximately 50% of the treated primary MS keratinocytes. We have also determined that the CMV promoter is capable of transiently expressing higher levels of  $\beta$ -galactosidase than the RSV and SV40 viral promoters. Future goals are to develop techniques to graft sheets of these transfected keratinocytes back onto the donor animal and determine how long the gene and its expression persist in the epidermis.

Similar techniques have been used to introduce the same DNA constructs into the epidermal keratinocytes of skin organ cultures and surprisingly, there is uptake and variable levels of expression in the organ culture epidermis which is very prominent around hair follicles in some experiments. We have grafted these *ex vivo* transfected organ cultures back onto the donor pig with plans to observe the expression of the introduced gene over time. For both of the *ex vivo* techniques described above, topical *in vivo* selection for keratinocytes containing the resistance gene can be performed after grafting the keratinocyte sheets or organ cultures back onto the pig. A selectable reagent, as described above, can be applied directly to the engrafted skin for selection over a period of time.

We are most excited about our direct *in vivo* approach of gene therapy. We have developed two different methods for direct DNA introduction into MS epidermis and have obtained expression in significant numbers of keratinocytes. To date, the expression of the  $\beta$ -galactosidase gene in these *in vivo* approaches is probably not stable and longterm, even though we have observed  $\beta$ -galactosidase activity for up to 4 weeks. The first approach involves the creation of epidermal blisters on MS skin by optimized amounts of suction and heat with a special apparatus we designed for this purpose. The blister perfectly separates the overlying epidermis from the underlying dermis. We then inject our DNA mixture directly into

the blister or into a special adhesive chamber we have designed to cover the blister. The goal is to transfect the DNA into the rapidly proliferating basal keratinocytes which are re-epithelializing the exposed dermis below the blister. The blister can be thought of as an *in vivo* culture dish. We find that both the re-epithelializing keratinocytes as well as the keratinocytes in the blister roof are capable of taking up DNA and expressing the  $\beta$ -galactosidase. The second approach is to inject the DNA mixture directly into the sub-epidermis (superficial dermis) with and without a special pretreatment of the skin called tape stripping which will be described below. Despite injection into the dermis, the DNA is readily taken up by all layers of the epidermis and expressed at high levels. Large numbers of keratinocytes can be transfected this way, and although we have not yet followed these animals long term, we suspect that we have not achieved significant amounts of the stable integration required for long-term expression. Current plans with these direct *in vivo* approaches include ongoing DNA dose-response titrations and kinetic studies of expression over time. We will begin to use the newly constructed plasmids containing resistance genes as well as the episomal plasmids. These selectable plasmids will allow us to begin long-term selection and enrichment.

As stated above, long-term expression requires the stable presence of the transfected DNA into stem cells or early precursors present in the basal keratinocyte layer of the epidermis. Methods such as tape stripping have been utilized to increase the likelihood that these early precursors or stem cells will divide and take up the injected DNA in this *in vivo* setting. Tape stripping is the physical removal of the outer layer of the epidermis called the stratum corneum. This procedure results in proliferation of the basal layer with frequent mitotic figures along with varying degrees of inflammation. Preliminary results suggest that mild amounts of tape stripping appear to increase the uptake and expression of DNA following direct injection *in vivo*, while excessive tape stripping seem to inhibit DNA uptake and expression, perhaps because of significant inflammation. These manipulations will be continued and titrated. Additionally, other agents exist, such as GM-CSF, which reportedly can induce proliferation of the basal layer, could be assessed.

Preliminary pilot experiments to assess the effects of G418 and Hygromycin B on the MS epidermis for topical *in vivo* selection have been very encouraging. These agents were applied in various concentrations both in liquid form using our special adhesive chambers and as special creams prepared by George Grimes in the Clinical Center Pharmacy. Even at relatively low doses, these agents appear to cause significant keratinocyte cell death over a few days. The doses are now being down-titrated to determine the right amount of mild selection over a prolonged period of time. Alternative selection approaches using genes such as the multi drug resistance (MDR) gene or the dihydrofolate reductase (dhfr) gene along with the appropriate selectable agents is also being planned.

We have also begun the cloning of the 5' flanking regulatory regions of endogenous keratin genes such as K14 and K5, utilizing partial sequences and restriction maps of the flanking regions. This is important because endogenous genes are felt to give better long term stable expression than viral promoters, which, although initially expressed at very high levels, appear to be inactivated or down-regulated over time when used in gene therapy. The regulatory regions of the keratin genes will also allow tissue specific expression of our inserted genes.

### Publications

None



## ANNUAL REPORT OF THE METABOLISM BRANCH

### SUMMARY OF SIGNIFICANT ACTIVITIES

#### NATIONAL CANCER INSTITUTE

October 1, 1992 through September 30, 1993

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 primary and acquired 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 focus on the definition of disorders in the control of the human immune response that underlie malignant and immunodeficiency diseases. Furthermore, they are directed toward developing rational approaches for the prevention and treatment of cancer, primary immunodeficiency diseases and AIDS. These studies include: 1) 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. 2) Somatic gene therapy for human genetic immunodeficiency diseases. 3) 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 and treating AIDS and cancer. 4) Identification, purification, and molecular genetic analysis of the multichain interleukin-2 receptor on normal and malignant lymphocytes. A major emphasis is placed on the development of different forms of IL-2 receptor-directed therapy. 5) Analysis of action of immunoregulatory cells including helper T cells, suppressor T cells, and macrophages that regulate antibody responses, and on studies of disorders of immunoregulatory cell interactions, in immunodeficiency diseases, in individuals with leukemias of these immunoregulatory cells.

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 non-neoplastic 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. Special emphasis is placed on the cellular receptors for normal growth factors, especially insulin-like growth factors I and II that participate in the hormonal control of normal and malignant growth.

#### MOLECULAR ANALYSIS OF TRANSACTING FACTORS THAT MEDIATE GENE EXPRESSION

Dr. Lou Staudt's laboratory focuses on the molecular cloning and characterization of novel lymphoid-restricted genes that regulate the development and function of lymphocytes.

Subtractive hybridization cDNA libraries have been used in conjunction with differential screening of cDNA libraries and automated DNA sequencing to isolate genes that are expressed predominantly in B and/or T lymphocytes. One lymphoid-restricted gene, Ly-GDI, encodes a protein bearing striking homology to a regulator of the ras-like G protein, rho. Ly-GDI inhibits the GTP/GDP exchange of rho and becomes phosphorylated during T-cell activation. The cloning of Ly-GDI suggests a mechanism by which signal transduction through the ras-like GTP binding proteins can be regulated in a cell type-specific fashion. Another lymphoid-restricted gene, JAW1, encodes a transmembrane protein which, surprisingly, resides in the endoplasmic reticulum. JAW1 has structural similarity to proteins involved in vesicle transport and fusion suggesting that these processes can be regulated in a lymphoid-restricted fashion. Subtracted cDNA libraries are being generated from normal human lymphocytes or human lymphoid malignancies such as Burkitt's lymphoma and chronic lymphocytic leukemia. Rapid and large scale automated DNA sequencing of such libraries has thus far resulted in the identification of over 30 novel lymphoid-restricted genes. The laboratory is currently characterizing in detail several of these novel genes which are predicted to encode nuclear proteins that may regulate gene expression in a lymphoid-specific fashion.

### SOMATIC GENE THERAPY FOR HUMAN GENETIC DISEASE

Michael Blaese's laboratory continues to focus on the development of gene therapy. He led the group which performed the first authorized use of gene transfer to treat human disease when they infused autologous ADA gene-corrected T cells into two girls with ADA deficiency SCID. Retroviral vectors were used to insert a normal human ADA gene into polyclonal peripheral blood T-cells which had been stimulated in tissue culture with an anti-T-cell receptor monoclonal antibody and IL-2. The gene-corrected T cells were culture expanded and then returned intravenously within 2 weeks to maintain a polyclonal repertoire. These ADA deficient patients have been treated 10-12 times over the past three years with such gene-corrected T cell infusions and are now showing signs of reconstituted immune reactivity including the production of isohemagglutinins and DTH in response to environmental antigenic stimulation. As the next phase in the development of this treatment, Dr. Blaese and his colleagues have used retroviral-mediated gene-transfer to insert a corrective ADA gene into CD34 selected lymphohematopoietic stem cells. GM-CSF was used to mobilize stem cells into the peripheral blood in a 12-year-old with ADA deficiency. Further, umbilical cord blood was used as a stem cell source in three newborn infants who had been diagnosed with ADA deficiency in utero. In these four cases, the gene-corrected cells were returned intravenously on Day 3.

A similar strategy of cellular immunotherapy has now been employed in a clinical trial in AIDS patients. Thirty seven (37) twin pairs discordant for HIV have been enrolled in a study to determine whether normal T-cells could be given to correct the immunodeficiency of AIDS patients and further, whether gene insertion could be used to introduce HIV resistance to these T cells.

Dr. Blaese has also been actively studying the use of direct gene transfer as a treatment for cancer. First he showed that tumor cells modified with the thymosine kinase (tk) gene

from herpes simplex could be readily killed by exposure to the antiherpes drug ganciclovir. Similarly, tumor cells modified to express the cytosine deaminase (Cda) gene from fungi could be killed when treated with the antifungal drug 5 flurocytosine. As a strategy for gene therapy, Dr. Blaese has implanted retroviral vector-producing fibroblasts into tumors locally to permit efficient gene transfer. In rats with brain tumors, this treatment was curative leading to the initiation of clinical trials using this strategy to test human glioblastoma or metastatic brain tumors.

### **MECHANISMS OF ANTIGEN-PRESENTATION AND T-LYMPHOCYTE RECOGNITION: APPLICATION TO VACCINE DESIGN**

Dr. Berzofsky has studied the mechanisms by which T cells recognize antigens presented 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 cancer. He addressed the molecular basis of peptide binding to MHC molecules and recognition by T cells, and has used this to design more potent vaccines. By studying synthetic peptides corresponding to antigenic epitopes, but with amino acid substitutions at different positions in the sequence, he identified mutations that produced higher affinity for the MHC molecule or for the T-cell receptor. One such peptide from the HIV envelope binds more tightly to a class II MHC molecule and is effective as a vaccine in mice at 10-100-fold lower doses than the natural sequence at eliciting helper T-cells specific for the natural HIV epitope. He also showed that only a few residues on the peptide or MHC molecule are critical for positive interaction, but other residues can interfere by causing adverse interactions, which he found play a major role in specific recognition. Dr. Berzofsky found that another HIV peptide is recognized by cytotoxic T lymphocytes (CTL) from 4 strains of mice with 4 different class I MHC molecules. Surprisingly, T cells of these 3 strains showed degenerate MHC restriction, recognizing the peptide with 3 different class I MHC molecules, and also used a limited number of T-cell receptor V-region genes. These findings provide a handle to understanding the presentation of this important peptide. This same HIV peptide is also presented by both class I and class II MHC molecules to CTL and helper T cells, respectively. Dr. Berzofsky's lab has compared the amino acid residues involved in presentation by each, and found a remarkable similarity, that may suggest a similar basis for binding. They have used these epitopes to make a synthetic peptide candidate vaccine for HIV, and have determined the optimal adjuvant formulation for a human phase I clinical trial. The toxicology and clinical protocols are being written. Dr. Berzofsky's lab has also developed a new method of immunizing with peptides without adjuvant, using dendritic cells. They have applied this to cancer vaccine development, to show that an endogenously expressed mutant p53 oncoprotein in a tumor cell can serve as a target antigen for CTL, and that such CTL can be elicited by immunization with a synthetic peptide from the mutant p53 sequence. They have also begun determining helper and CTL responses to peptides from human papillomavirus oncoproteins E6 and E7, which should be useful for both diagnosis and possible vaccine treatment of papillomavirus-related cervical cancer. In addition, they have found that the immune defect in asymptomatic HIV-infected patients in their T-cell response in vitro to HIV peptides can be overcome by use of anti-IL-10 antibodies, suggesting a possible approach to therapy.

## THE MULTICHAIN IL-2 RECEPTOR: MOLECULAR CHARACTERIZATION AND USE AS A TARGET FOR IMMUNOTHERAPY

Effective therapy of cancer using antibody mediated therapy has been elusive. A number of factors explain the low therapeutic efficacy observed. Unmodified monoclonal antibodies are immunogenic and elicit a human immune response to the murine antibody. Moreover, mouse monoclonal antibodies are not cytotoxic against neoplastic cells in humans and in most cases are not directed against a vital cell surface structure such as a receptor for a growth factor required for tumor cell proliferation. Dr. Thomas Waldmann has addressed these issues by using the IL-2 receptor as a target for monoclonal antibody immunotherapy, by genetic engineering to create less immunogenic and more effective monoclonal antibodies, and by arming such antibodies with toxins or radionuclides to enhance their effector action. Dr. Waldmann previously identified two peptides that bind IL-2: the 55 kD protein IL2R $\alpha$  chain reactive with the anti-Tac monoclonal antibody, and the 70/75 kD IL-2R $\beta$  protein reactive with a monoclonal antibody termed Mik $\beta$ 1. He proposed a multichain model for the high affinity receptor in which both IL-2R $\alpha$ - and IL-2R $\beta$ -binding proteins are associated in a receptor complex. A third component IL-2R $\gamma$  was identified by the laboratory of Dr. Kazuo Sugamura in Japan. Dr. Waldmann has recently identified a fourth component of the IL-2 receptor, a 30 kD peptide that is modulated from the surface of activated T cells by the addition of interleukin-2.

Dr. Nelson and his colleagues 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 and urine of normal individuals *in vivo*. Elevated levels of this receptor are present in a variety of malignancies of the lymphoreticular system. In patients with adult T-cell leukemia (ATL), reductions in serum levels of sIL-2R correlated with responses to chemotherapy. In collaboration with Dr. Thomas Waldmann this has also been shown in ATL patients receiving IL-2R-directed therapies. Elevated serum levels of sIL-2R were also observed in patients with the acquired immune deficiency syndrome (AIDS) and carriers of the human immunodeficiency virus type 1. Thus, the measurement of sIL-2R is useful in the management of patients with immunologic activation *in vivo*.

Dr. Waldmann designed a novel form of therapy, IL-2 receptor directed therapy, to exploit the difference in IL-2 receptor expression between normal resting cells and abnormal T cells that cause disease. Initially Dr. Waldmann focused his IL-2 receptor directed therapeutic studies on patients with adult T-cell leukemia (ATL). ATL is an aggressive disorder with no known curative chemotherapy that kills patients on average in 20 weeks. All populations of leukemic cells examined by Dr. Waldmann from patients with HTLV-I-associated ATL express very large numbers of IL-2 receptors identified by the anti-Tac monoclonal antibody. Dr. Waldmann initiated a therapeutic trial using the unmodified murine anti-Tac monoclonal antibody in the treatment of patients with ATL with the goal of preventing the interaction of IL-2 with the IL-2 receptor thus depriving the malignant cells of a growth factor required for their proliferation and survival. The patients studied did not suffer any toxicity. Seven of the 19 patients studied underwent a remission; in three cases a complete remission lasting from 8 to over 36 months following initiation of anti-Tac therapy. Although use of such murine antibodies is of value in the therapy of human diseases, their

effectiveness is limited by the fact that rodent monoclonal antibodies often induce a human immune response to them. To circumvent this difficulty genetically engineered antibody variants of anti-Tac were produced by combining the rodent genetic elements encoding the hypervariable regions with human, constant and framework region genes. Dr. Waldmann showed that the humanized version of the anti-Tac monoclonal antibody is dramatically less immunogenic than the parent mouse monoclonal. Furthermore, he showed that the humanized version of anti-Tac manifests a killing ability directed toward human tumor cells termed antibody-dependent cellular cytotoxicity that is absent in the parental mouse anti-Tac. With the lowered immunogenicity, improved pharmacokinetics, and a new effector function antibody-dependent cellular cytotoxicity it is hoped that there will be a substantial improvement in the therapeutic efficacy of this genetically engineered monoclonal antibody. Dr. Waldmann confirmed this predicted improved effectiveness in preclinical animal models and has initiated therapeutic trials with humanized anti-Tac in patients with IL-2 receptor expressing malignancies. The clinical trial with this antibody involves patients with IL-2 receptor-expressing leukemia and lymphoma as well as individuals undergoing graft versus host disease.

Drs. Waldmann and David Nelson extended the clinical therapeutic implications of monoclonal antibodies by focusing on the use of these agents as carriers of cytotoxic agents. Here the goal is to maintain the specificity of the monoclonal antibody while increasing its capacity to kill unwanted cells by coupling toxins or radionuclides to it. They developed cytotoxic agents wherein  $\alpha$ - and  $\beta$ -emitting radionuclides are conjugated to anti-Tac by use of bifunctional chelates. For example Dr. Waldmann showed that bismuth-212, an  $\alpha$ -emitting radionuclide conjugated to anti-Tac was well-suited for a therapeutic role. In parallel studies Drs. Waldmann and Nelson bound the  $\beta$ -emitting radionuclide Yttrium-90 to anti-Tac using chelates that neither damage the antibody nor permit the elution of radiolabeled Yttrium from it. Following efficacy and toxicity studies in animal models, they initiated a dose escalation trial with Yttrium-labeled anti-Tac for the treatment of HTLV-I-associated adult T-cell leukemia (ATL). Eleven of the 17 patients underwent a partial or complete remission following Yttrium-90 anti-Tac therapy. One of the limitations of this approach was related to the immunogenicity of the murine antibody used in this trial. To circumvent this problem a therapeutic trial has been initiated with  $^{90}\text{Y}$ -humanized anti-Tac in lieu of the radiolabeled murine monoclonal antibody.

## **INSULIN-LIKE GROWTH FACTOR (IGF-I AND IGF-II) RECEPTORS**

Dr. Peter Nissley is studying the mechanism of action of insulin-like growth factors (IGF-I and IGF-II), focusing on the function of the two receptors that bind these ligands, the IGF-I receptor and the IGF-II/mannose 6-phosphate receptor. Compared to what is known about the signaling pathway of other growth factor receptors with intrinsic tyrosine kinase activity, relatively little is known about the immediate postreceptor events for the IGF-I receptor. Dr. Nissley's laboratory asked whether activation of extracellular signal-regulated kinases (ERKs) 1 and 2 (also called MAP kinases) occurs following activation of the IGF-I receptor in MG-63 human osteosarcoma cells. ERK1 and ERK2 have been implicated as being important serine/threonine kinases in the signaling pathway leading to growth and differentiation. ERK1 and ERK2 require phosphorylation on tyrosine and threonine for activation. Using

immunoprecipitation of cell lysates with anti-phosphotyrosine-Agarose, it was demonstrated that addition of IGF-I to serum-starved MG-63 cells resulted in rapid phosphorylation of a 45 kDa species identified by Western blotting as a MAP kinase. Resolution of ERK1 and ERK2 by ion exchange HPLC chromatography (Mono Q) and analysis of column fractions by immunoblotting with anti-MAP kinase and antiphosphotyrosine antibodies showed that ERK2 was activated by IGF-I. Thus it is likely that one of the members of the MAP kinase family is involved in the signaling pathway for the IGF-I receptor.

Dr. Nissley's laboratory has continued studies on fibroblasts from two patients with deletion of the distal long arm of one copy of chromosome 15. These patients are of interest because severe growth retardation is a feature of the syndrome and the IGF-I receptor gene is located in the deleted segment of chromosome 15. Using a solution hybridization/nuclease protection assay to measure levels of IGF-I receptor mRNA, it was possible to demonstrate significantly decreased levels of mRNA in one of the patient's fibroblasts compared to age-matched control fibroblasts. In the second patient the level of mRNA was not significantly different from the levels in control fibroblasts. To further assess IGF-I receptor function Dr. Nissley's laboratory examined the stimulation of [<sup>3</sup>H]thymidine incorporation into DNA by a full range of IGF-I concentrations. There was no significant difference between fibroblasts from patients and controls for ED<sub>50</sub> for IGF-I. From these experiments and earlier ones, Dr. Nissley concluded that fibroblasts from these two patients with chromosome 15q deletion syndrome exhibit decreased cell surface expression of the IGF-I receptor but there is no evidence for IGF-I receptor functional impairment in two different bioassays. Thus, results from the first examination of fibroblasts from patients with this genetic disorder do not support the hypothesis that the growth impairment exhibited by these patients results from having only one copy of the IGF-I receptor gene.

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

PROJECT NUMBER

Z01 CD-04002-24 MET

PERIOD COVERED

October 1, 1992 through September 30, 1993

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

Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunctions

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

Thomas A. Waldmann, M.D.	Branch Chief	MET, NCI
Jack D. Burton, M.D.	Expert	MET, NCI
Carolyn K. Goldman	Microbiologist	MET, NCI
Angus Grant, Ph.D.	Senior Staff Fellow	MET, NCI
Frank Hartmann, M.D.	Fogarty Visiting Associate	MET, NCI
Claude Kasten-Sportes, M.D.	Senior Staff Fellow	MET, NCI
Christian Peters, M.D.	Fogarty Visiting Associate	MET, NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, NCI  
 Medicine Branch, NCI  
 Radiation Oncology Branch, NCI

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

13

PROFESSIONAL:

11

OTHER:

2 B 100%

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.)

Dr. Waldmann developed IL-2 receptor-directed therapy for patients with leukemia. The scientific basis for this approach is provided by his observation that resting T-cells do not express IL-2 receptors but receptors are expressed by the abnormal T-cells of patients with lymphoma/leukemia, those with select forms of autoimmune disease, and individuals rejecting allografts. Dr. Waldmann proposed a multichain model for the high affinity IL-2 receptor involving two IL-2 binding proteins: a 55 kD (IL-2R $\alpha$ ) and a 75 kD (IL-2R $\beta$ ) protein. To exploit the difference in IL-2 receptor expression between normal and malignant cells, he has initiated IL-2 receptor directed therapy in patients with human lymphotropic virus I (HTLV-I) associated adult T-cell leukemia (ATL). Using unmodified anti-Tac monoclonal antibody that reacts with IL-2R $\alpha$ , one-third of the patients with ATL treated have undergone a remission. There was no toxicity observed. However, unmodified monoclonal antibodies are limited by their immunogenicity and their poor effector functions. To address these issues "humanized" anti-Tac was produced that retains the complementarity-determining regions from the mouse with the remainder of the molecule derived from human IgG1. This antibody is dramatically less immunogenic than the murine version and, in contrast to the parent antibody, manifests antibody-dependent cellular cytotoxicity. A clinical trial with this antibody has been initiated in patients with IL-2 receptor-expressing leukemias and lymphomas as well as individuals with corticosteroid-resistant graft-versus-host disease. To enhance its effector function anti-Tac was armed with toxins and  $\alpha$ - and  $\beta$ -emitting radionuclides. In a clinical trial of  $^{90}\text{Y}$ -anti-Tac in ATL 11 of the 17 patients with ATL underwent a partial or complete remission. Thus, the clinical application of IL-2 receptor-directed therapy represents a new perspective for the treatment of certain neoplastic diseases.

## Continuation Sheet for PHS 6040

Professional Personnel, Continued:

Erich Roessler, M.D., Ph.D.	Senior Staff Fellow	MET, NCI
Arthur Sleeper, Ph.D., M.D.	Clinical Associate	MED, NCI
Sikiru A. Tinubu, M.D.	Visiting Associate	MET, NCI
Sara L. Zaknoen, M.D.	Clinical Associate	MET, NCI

Project DescriptionMajor Findings:

Effective therapy of cancer using unmodified monoclonal antibody mediated therapy has been elusive. A number of factors explain the low therapeutic efficacy observed. Unmodified monoclonal antibodies are immunogenic and elicit a human immune response to the murine antibody. Moreover, mouse monoclonal antibodies are not cytotoxic against neoplastic cells in humans and in most cases are not directed against a vital cell surface structure such as a receptor for a growth factor required for tumor cell proliferation. Dr. Waldmann has addressed these issues by using the IL-2 receptor as a target for monoclonal antibody immunotherapy, by genetic engineering to create less immunogenic and more effective monoclonal antibodies, and by arming such antibodies with toxins or radionuclides to enhance their effector action. Dr. Waldmann previously identified two peptides that bind IL-2: the 55 KD protein IL2R $\alpha$  reactive with the anti-Tac monoclonal antibody, and the 70/75 kD IL-2R $\beta$  protein reactive with a monoclonal antibody termed Mik $\beta$ 1. He proposed a multichain model for the high affinity receptor in which both IL-2R $\alpha$ - and IL-2R $\beta$ -binding proteins are associated in a receptor complex. Dr. Waldmann recognized the value of the IL-2 receptor as a therapeutic target. Normal resting T cells, B cells and monocytes do not express the IL-2 receptor. In contrast, this receptor is expressed by the abnormal cells of patients with certain forms of cancer or autoimmune disease and those rejecting allografts. Dr. Waldmann designed a novel form of therapy, IL-2 receptor directed therapy, to exploit this difference in IL-2 receptor expression between normal resting cells and abnormal T cells that cause disease. Initially, Dr. Waldmann focused his IL-2 receptor directed therapeutic studies on patients with adult T-cell leukemia (ATL). ATL is an aggressive disorder with no known curative chemotherapy that kills patients on average in 20 weeks. All populations of leukemic cells examined by Dr. Waldmann from patients with HTLV-I-associated ATL express very large numbers of IL-2 receptors identified by the anti-Tac monoclonal antibody. Dr. Waldmann initiated a trial using unmodified anti-Tac monoclonal antibody for the treatment of patients with ATL with the goal of preventing the interaction of IL-2 with the IL-2 receptor thus depriving the malignant cells of a growth factor required for their proliferation and survival. The patients studied did not suffer any toxicity. Seven of the 19 patients studied underwent a remission; in three cases a complete remission lasting from 8 to over 36 months following initiation of anti-Tac therapy. Patients who do not respond to anti-Tac still express the interleukin-2 receptor but do not produce IL-2 nor require this lymphokine for proliferation. Dr. Waldmann has identified several alterations in the leukemic cells of such patients including mutations in the IL-2R $\beta$  subunit, a change in the IL-2R-associated src-type tyrosine kinase from lck to lyn and the production of a previously undefined lymphokine. Although murine antibodies are of value, their effectiveness is limited by the fact that rodent monoclonal antibodies often induce a human immune response to them. To circumvent this difficulty genetically engineered antibody variants of anti-Tac were produced by combining the rodent genetic elements encoding the hypervariable regions with human, constant and framework region genes. Dr. Waldmann showed that the humanized version of the anti-Tac monoclonal antibody is dramatically less immunogenic than the parent mouse monoclonal. Furthermore, he showed that the humanized version of anti-Tac manifests a killing ability directed toward human tumor cells termed antibody-dependent cellular cytotoxicity that is absent in the parental mouse anti-Tac. With the lowered



immunogenicity, improved pharmacokinetics, and a new effector function antibody-dependent cellular cytotoxicity, it is hoped that there will be a substantial improvement in the therapeutic efficacy of this genetically engineered monoclonal antibody. Dr. Waldmann confirmed this predicted improved effectiveness in preclinical animal models and has initiated therapeutic trials with humanized anti-Tac in patients with IL-2 receptor expressing malignancies. In parallel studies a humanized version of Mik $\beta$ 1 that blocks binding to the IL-2R $\beta$  component has been generated by combining the complementarity determining regions of Mik $\beta$ 1 with human immunoglobulin framework and constant regions. As with anti-Tac humanized Mik $\beta$ 1 manifests antibody-dependent cellular cytotoxicity. Furthermore, humanized Mik $\beta$ 1 that blocks the interaction of IL-2 with the IL-2R $\beta$  subunit complements the anti-IL-2R $\alpha$  chain antibody anti-Tac in inhibiting IL-2 induced proliferation.

Dr. Waldmann extended the clinical therapeutic implications of monoclonal antibodies by focusing on the use of these agents as carriers of cytotoxic agents. Here the goal is to maintain the specificity of the monoclonal antibody while increasing its capacity to kill unwanted cells by coupling toxins or radionuclides to it. He developed alternative cytotoxic agents wherein  $\alpha$ - and  $\beta$ -emitting radionuclides are conjugated to anti-Tac by use of bifunctional chelates. For example he showed that bismuth-212, an  $\alpha$ -emitting radionuclide conjugated to anti-Tac was well-suited for a therapeutic role. In parallel studies he bound the  $\beta$ -emitting radionuclide Yttrium-90 to anti-Tac using chelates that neither damage the antibody nor permit the elution of radiolabeled Yttrium from it. Following efficacy and toxicity studies in animal models, he initiated a dose escalation trial with Yttrium-labeled anti-Tac for the treatment of HTLV-I-associated adult T-cell leukemia (ATL). Eleven of 17 patients with ATL studied underwent a partial or complete remission. It is hoped that Yttrium-90 chelated to humanized anti-Tac and to humanized Mik $\beta$ 1 will prove to be effective, relatively nontoxic agents for the treatment of an array of human leukemias. Thus the new insights concerning the IL-2/IL-2 receptor system are providing a novel perspective for the treatment of certain neoplastic diseases. A therapeutic trial using  $^{90}\text{Y}$ -humanized anti-Tac in the treatment of patients with ATL has been initiated.

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

PROJECT NUMBER

Z01 CB 04015-4 MET

PERIOD COVERED

October 1, 1992 through September 30, 1993

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

**Development and Function of Humoral and Cellular Immune Mechanisms**

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

R. Michael Blaese, M.D.	Section Chief	MET, NCI
Fabio Candotti, M.D.	Special Volunteer	MET, NCI
Kenneth Culver, M.D.	Medical Officer, Spec. Vol.	MET, NCI
Thomas Felzmann, M.D.	Special Volunteer	MET, NCI
Richard Hess, Ph.D.	Commissioned Officer	MET, NCI
Hiroyuki Ishii, M.D., Ph.D.	Visiting Fellow	MET, NCI
Kimberly Leightling, Ph.D.	Senior Staff Fellow	MET, NCI
Ruben Moreno, M.D.	Special Volunteer	MET, NCI

COOPERATING UNITS (if any)

E. Clifford Lane, M.D.	Clinical Director	NIAID
Edward Oldfield, M.D.	Chief	SNB, NINDS
Gene Shearer, Ph.D.	Section Chief	EIB, NCI

LAB/BRANCH

Metabolism Branch

SECTION

Cellular Immunology

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

11

PROFESSIONAL:

8

OTHER:

3 B 100%

CHECK APPROPRIATE BOXES)

(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.)

Michael Blaese's laboratory continues to focus on the development of gene therapy. He led the group which performed the first authorized use of gene transfer to treat human disease when they infused autologous ADA gene-corrected T cells into two girls with ADA deficiency SCID. Retroviral vectors were used to insert a normal human ADA gene into polyclonal peripheral blood T cells which had been stimulated in tissue culture with an anti-T cell receptor monoclonal antibody and IL-2. The gene-corrected T cells were culture expanded and then returned intravenously within 2 weeks to maintain a polyclonal repertoire. These ADA deficient patients have been treated 10-12 times over the past three years with such gene-corrected T cell infusions and are now each showing signs of reconstituted immune reactivity including the production of isohemagglutinins and DTH in response to environmental antigenic stimulation. As the next phase in the development of this treatment, Dr. Blaese and his colleagues have used retroviral-mediated gene-transfer to insert a corrective ADA gene into CD34 selected lymphohematopoietic stem cells. GM-CSF was used to mobilize stem cells into the peripheral blood in a 12-year-old with ADA deficiency. Further, umbilical cord blood was used as a stem cell source in three newborn infants who have been diagnosed with ADA deficiency in utero. In these four cases, the gene-corrected cells were returned intravenously on Day 3. Dr. Blaese's laboratory has also developed a unique new approach to direct gene therapy of cancer using inoculation of murine fibroblasts producing retroviral vectors directly into tumors in situ. Using vectors containing the gene for herpes simplex thymidine kinase, he has shown cure of brain tumors in rats following systemic administration of the anti-herpes virus drug, ganciclovir. This antitumor effect was shown to be aided by a "bystander effect" in which phosphorylate ganciclovir is transmitted from tk-gene containing tumor cells to neighboring unmodified tumor cells through "gap junctions" extending delivery of toxin widely in the treated tumors.

Professional Personnel, Continued

Craig Mullen, M.D., Ph.D.	Commissioned Officer	POB, NCI
Jay Ramsey, M.D., Ph.D.	Clinical Associate	MET, NCI
Renaud Touraine, M.D.	Special Volunteer	MET, NCI

Project DescriptionMajor Findings:

A major effort of the Cellular Immunology Section for the past several years has been directed toward the development of techniques of gene transfer for application to clinical gene therapy. In 1987 we began to study the possibility of employing T lymphocytes as cellular vehicles for clinical gene transfer. We had already shown that the metabolic defect in T-cell lines from patients with ADA deficiency could be cured by retrovirus-mediated gene transfer. T cells are readily available in the peripheral blood, readily adapt to tissue culture manipulation, and will stably accept transferred genes. In addition, immune T cells can be very long lived as evidenced by the observation that adults maintain DTH and antibody to antigens such as tetanus toxoid for decades after their initial immunization. We first demonstrated that the hADA gene could be introduced into antigen-specific murine CD4 T cells in vitro and that these gene-modified cells would persist in recipient mice for several months and continue to express the introduced hADA gene. We then showed that T cells cultured from monkey blood or lymph node could be successfully transduced with a foreign gene and that these gene-modified T cells would persist for up to 2 years when reintroduced into the autologous monkeys.

As an initial application of gene transfer in a clinical situation based on these findings, we established a collaboration with Steven Rosenberg of the NCI Surgery Branch. This gave us the opportunity to evaluate the consequences of retrovirus mediated gene transfer in patients with terminal cancer and a limited life expectancy who were already being treated with lymphocyte infusions. Our study used the NeoR gene to label tumor infiltrating lymphocytes (TIL) so that their survival and distribution in the body could be determined to see if this might correlate with the anticancer effect. We were able to show that TIL remain in the peripheral blood for about 3 weeks after a single iv infusion and that they localize to the sites of tumor metastases within 2-3 days in patients who experience subsequent remission. Importantly, these studies also demonstrated that retroviral-mediated gene transfer into lymphocytes could be successfully employed in patients and that no untoward consequences at all were observed in the recipients of the gene-modified cells.

With the experience of this successful clinical application of gene transfer behind us, we next moved on to the initial use of gene transfer for the treatment of human disease, true gene therapy. In our studies of children with ADA deficiency SCID, it was shown that unexpectedly we grew polyclonal T cells from their peripheral blood if a combination of anti-TCR monoclonal antibody (OKT3) and IL2 was used to stimulate T cell proliferation. We also demonstrated that we could successfully insert the corrective ADA gene into these proliferating non-transformed T cells using retroviral vectors and that the inserted gene was expressed and the transduced cells produced normal quantities of adenosine deaminase enzyme which was functionally active. On September 14, 1990, the first authorized gene therapy experiment began with the treatment of a 4-year-old girl with ADA deficiency. Subsequently a second child has been enrolled in the protocol and both are doing very well. The patient's T cells are collected periodically from their peripheral blood by apheresis, cultured to expand their number by 100-fold while the ADA gene is inserted, and then reinfused intravenously. To date, the first patient has received 11 infusions and the second patient 12 treatments. The peripheral T-cell count is now in the normal range for each child. Each is now also producing normal

amounts of antibodies to red blood cells (isohemagglutinins), responses which were deficient before treatment began and enhanced T-cell function and DTH. We will continue to extensively evaluate the immune function in these patients over the next several years as well as enroll additional patients into the study in the coming months.

A similar strategy of cellular immunotherapy has now been employed in a clinical trial in AIDS patients. Thirty seven (37) identical twin pairs discordant for HIV have been enrolled in a study to determine whether normal T-cells could be given to correct the immunodeficiency of AIDS patients and further, whether gene insertion could be used to introduce HIV resistance to these T cells. Our laboratory has also been actively studying the use of direct gene transfer as a treatment for cancer. First we showed that tumor cells modified with the thymidine kinase (tk) gene from herpes simplex could be readily killed by exposure to the antiherpes drug ganciclovir. Similarly, tumor cells modified to express the cytosine deaminase (Cda) gene from fungi could be killed when treated with the antifungal drug 5 flurocytosine. As a strategy for gene therapy, we have implanted retroviral vector-producing fibroblasts into tumors locally to permit efficient gene transfer. In rats with brain tumors, this treatment was curative leading to the initiation of clinical trials using this strategy to treat human glioblastoma or metastatic brain tumors.

Work has also continued on our long-term interest in the Wiskott-Aldrich syndrome with studies of platelet function before and after splenectomy, detailed lymphocyte phenotype analysis of both T- and B-lymphocytes, family studies for linkage analysis to attempt to accurately identify the location of the gene on the X chromosome, and studies of the pattern of unbalanced X-chromosome inactivation in the blood lymphocytes and myeloid cells of the carriers of this disorder. In brief, our linkage studies indicate that we are within 1 CM of the gene locus on the X chromosome. In collaboration with S. P. Kwan, we have overlapping YAC clones spanning this entire region of the chromosome so that work is well along on the final cloning and identification of the WAS gene. In collaboration with G. Shearer and M. Clerici, we have also discovered a previously unrecognized defect in the antigen presentation capacity of cells from WAS patients which should help us identify the genetic defect. WAS-APC cells are unable to present exogenous peptide antigens in association with Class I MHC determinants which is related to instability of the cell surface complex consisting of antigen, MHC Class I, and  $\beta_2$  microglobulin. This defect is corrected by the addition of exogenous  $\beta_2$  microglobulin. Studies are in progress to more fully delineate the mechanism underlying this abnormality and to define its molecular basis.

Our studies of the compound succinylacetone (SA) have also continued to provide insights to this very potent immunosuppressive material. SA is a 7 carbon organic acid which was originally studied because it is an inhibitor of the second step of heme biosynthesis. It has very broad immunosuppressive activity on both T and B cell function. It prevents cardiac and skin allograft rejection in rats. SA used as the sole immunosuppressive agent prolongs the survival of cardiac transplants in monkeys and miniature swine for as long as the drug is administered (at least 2 months). SA treatment prevents GVHD in rats given total allogeneic bone marrow transplants and yet permits stable long term engraftment. It is the most effective agent yet tested in preventing acute GVHD in lethally irradiated dogs given totally mismatched BMT. In rats SA completely blocks the primary antibody response to T cell independent as well as T cell dependent antigens. It inhibits antibody production in miniature swine and primates as well. SA treatment has no effect on the generation of a normal (non-immune) inflammatory response or on granulocyte function. The drug is effective in preventing experimental autoimmune uveitis and will reverse ongoing autoimmune "adjuvant arthritis." Treatment with immunosuppressive doses of SA does not inhibit the appearance of early T-cell activation antigens. Its effect is not reversed by addition of growth factors such as IL2. It does inhibit the in vitro proliferative responses of T cells to mitogen or antigen stimulation, but only at doses which are 10-100 fold higher than those achieved in vivo. We have recently shown that although it does not inhibit antigen induced responses or the MLC in primary culture stimulation, secondary stimulation of these cells in vitro is totally inhibited. This new finding provides us with a measurable in vitro effect which should assist in the definition of the mechanism of action of the drug.

Honors and Awards:

Elected to membership in the Association of American Physicians

The Inaugural Alexandra J. Kefalides Memorial Lecture, University of Pennsylvania School of Medicine

Distinguished Medical Scientist Lectureship, Ohio State University MD/PhD Program

Markey Molecular Medicine Lectureship, University of Washington

Inaugural Keynote Lecture, University of Minnesota Medical School, MD/PhD Student Research Festival

Publications:

Blaese RM. Amendment to clinical research project: treatment of severe combined immunodeficiency disease (SCID) due to adenosine deaminase deficiency with CD34<sup>+</sup> selected autologous peripheral blood cells transduced with a human ADA gene. *Human Gene Therapy*, in press.

Blaese RM. Development of gene therapy for immunodeficiency: adenosine deaminase deficiency. *Pediatric Res* 1993;33(suppl)S49-S55.

Blaese RM. Progress in gene therapy for ADA deficiency and cancer. *Brit J Haematol*, in press.

Blaese RM, Culver KW. Gene therapy for primary immunodeficiency. *Immunodeficiency Rev* 1992;3:329-49.

Blaese RM, Culver KW. Gene therapy for primary immunodeficiency disease. In: Rosen FS, Seligmann M, eds. *Immunodeficiencies*. Chur: Harwood 1993;727-41.

Blaese RM, Culver KW, Miller AD, Anderson WF. Gene therapy for immunodeficiency and cancer. *Progress in Immunology* 1993;VIII:553-55.

Blaese RM, Mullen CA, Culver KW. Progress in gene therapy for immunodeficiency and cancer. *Proc Nippon-Roche Symposium on Molecular Medicine*, in press.

Blaese RM, Parkman R. Combined immunodeficiency diseases. In: Scriver DR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic basis of inherited disease*, 7th ed. New York: McGraw-Hill, in press.

Cornetta C, Nguyen N, Morgan RA, Muenchau DD, Hartley JW, Blaese RM, Anderson WF. Infection of human cells with murine amphotropic replication-competent retroviruses. *Virology*, in press.

Culver KW, Anderson WF, Blaese RM. Gene therapy. *Encyclopaedia Britannica: 1993 Yearbook of Science* (1992). Chicago: Encyclopaedia Britannica 1993;126-37.

Culver KW, Blaese RM. Gene therapy for immunodeficiency disease. *New concepts in immunodeficiency diseases*. London: John Wiley 1993;427-56.

Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM. In vivo gene transfer with retroviral vector producer cells for treatment of experimental brain tumors. *Science* 1992;256:1550-52.

Mullen CA, Anderson KD, Blaese RM. Splenectomy and/or bone marrow transplantation in the management of the Wiskott-Aldrich Syndrome: long-term follow-up of 62 cases. *Blood*, in press.

Mullen CA, Coale MM, Levy AT, Stetler-Stevenson WG, Liotta LA, Brant S, Blaese RM. Fibrosarcoma cells transduced with the IL-6 gene exhibit reduced tumorigenicity, increased immunogenicity and decreased metastatic potential. *Cancer Res* 1992;52:6020-24.

Ram Z, Culver KW, Walbridge S, Blaese RM, Oldfield EH. In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res* 1993;53:1-6.

Winkelstein A, Hess RA, Blaese RM. Inhibition of human lymphoproliferative responses and altered lymphocyte membrane phenotype by succinylacetone. *Immunopharmacology* 1992;24:161-70.

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

PROJECT NUMBER

Z01 CB 04016-20

PERIOD COVERED

October 1, 1992 through September 30, 1993

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)

S. Peter Nissley, M.D.	Senior Investigator	MET, NCI
Daisy De Leon, M.D.	Senior Staff Fellow	MET, NCI
Wlodzimierz Lopaczynski, M.D.	Fogarty Visiting Associate	MET, NCI
Mustafah Saad-El-Deen, M.D.	Visiting Associate	MET, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolism Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

6.0

PROFESSIONAL:

3.7

OTHER:

2.3 B 100%

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.)

A rapid, sensitive, dot-blot assay for insulin-like growth factor-II (IGF-II) based on chemiluminescence methodology has been further evaluated for possible interference by IGF binding proteins (IGFBPs). Six different IGFBPs were tested in the dot blot assay at concentrations up to 60-fold the concentration of IGF-II. The IGF-II signal was not diminished. By contrast, in a conventional radioimmunoassay using the same monoclonal antibody to IGF-II, the binding proteins produced significant interference, causing 23% to 87% competition of binding of <sup>125</sup>I-IGF-II to the antibody. Thus the IGF-II dot blot assay is unique in allowing direct measurement of IGF-II in conditioned medium from cells in culture without interference by IGFBPs.

Addition of IGF-I to MG-63 human osteosarcoma cells resulted in rapid activation of extracellular signal regulated kinase 2 (ERK2) as assessed by ion exchange HPLC and ERK kinase antibodies. ERK1 appeared to be already activated in serum-starved MG-63 cells and not to be further activated following addition of IGF-I. Thus ERK2 activation may be part of the signaling pathway for the IGF-I receptor.

Further evaluation of fibroblasts from two patients with deletion of the distal long arm of chromosome 15 showed evidence for decreased expression of IGF-I receptor mRNA in one of the patients compared to age-matched controls. Evaluation of receptor function in a bioassay which measured the incorporation of [<sup>3</sup>H]thymidine into DNA in the presence of a full range of IGF-I concentrations showed no difference in the ED<sub>50</sub> for IGF-I between the patient and control fibroblasts. We conclude from these findings and our earlier results that although there is evidence for decreased IGF-I receptor expression in fibroblasts from patients with chromosome 15q deletion syndrome, receptor function as assessed by cellular response to IGF-I is not impaired.



## Project Description

### Major Findings:

#### Transfection of MCF-7 human breast cancer cells with the IGF-II gene.

We are testing our hypothesis that in a cell that produces IGF-II the targeting of lysosomal enzymes will be modified so that flux through the intracellular pathway to lysosomes is decreased and the traffic through the secretory pathway is increased. This prediction follows from our finding that IGF-II inhibits the cellular uptake of lysosomal enzymes by inhibiting binding to the receptor. We are transfecting the MCF-7 breast cancer cell line with vectors containing IGF-II cDNA inserts together with a vector containing the multiple drug resistance gene (*mdr*). Under selective pressure the *mdr* gene undergoes gene amplification. The expectation is that the IGF-II gene will also be amplified resulting in increased IGF-II production. We have constructed two IGF-II expression vectors by inserting a PCR product corresponding to the mature M<sub>r</sub> 7471 IGF-II containing B, C, A, and D domains and inserting a second PCR product corresponding to a large precursor form of IGF-II containing B, C, A, D, and E domains. This IGF-II species has been shown to undergo glycosylation resulting in an even larger IGF-II species which might be expected to be more effective in inhibiting the binding of lysosomal enzymes to the receptor is the mechanism is by steric inhibition. The IGF-II inserts were generated by attaching restriction enzyme sites to oligonucleotide primers and generating PCR products using as template a human IGF-II cDNA provided by Graham Bell. The PCR products were cut with restriction enzymes and inserted into the cloning cassette of pRC/CMV, a eukaryotic expression vector which includes a human cytomegalovirus promoter, a human growth hormone polyadenylation signal, and a neomycin gene for selection of stable transfectants. The IGF-II base sequences in the IGF-II expression vectors were confirmed by DNA sequencing. These expression vectors together with an *mdr* expression plasmid have recently been used to transfect MCF-7 cells; these cultures are currently being subjected to selective pressure to isolate transfectants.

The identification of transfected clones that produce large amounts of IGF-II requires a convenient method for the rapid detection of IGF-II in small amounts of multiple samples of conditioned medium. We have developed a dot-blot assay based on Amersham enhanced chemiluminescence (ECL) methodology which detects picogram quantities of IGF-II. A persistent problem in the assay of IGFs by competitive binding techniques has been interference by IGF binding proteins (IGFBPs), six of which have been described to date. To evaluate interference by IGFBPs, 0.15 ng to 5 ng of IGFBPs 1-6 were incubated with 0.08 ng of IGF-II and then dot blotted and assayed. None of the IGFBPs diminished the IGF-II signal. Maximal binding of IGF-II to the IGFBPs as determined by the charcoal method ranged from 23% (IGFBP-5) to 87% (IGFBP-2, IGFBP-3). In contrast, significant interference by the IGFBPs was observed when the same concentrations of IGFBPs and <sup>125</sup>I-IGF-II were used in a radioimmunoassay which used the same monoclonal antibody. Maximal inhibition of <sup>125</sup>I-IGF-II binding to the antibody by IGFBPs ranged from 34% (IGFBP-4) to 79% (IGFBP-2, IGFBP-3). Why IGF-II is detected in the dot blot assay without IGFBP interference is not understood. We speculate that the conformation of the IGF-II: binding protein complex may be altered by binding to the nitrocellulose, exposing the IGF-II epitope that is recognized by the antibody.

MCF-7 cells secrete large amounts of the lysosomal enzyme, cathepsin D. To analyze the distribution of cathepsin D in the MCF-7 cells that have been transfected with IGF-II we have developed a polyclonal antibody against human cathepsin D that can be used for immunoprecipitation of metabolically labeled cathepsin D. This antiserum was developed by purifying cathepsin D to homogeneity from human placenta and immunizing rabbits. This polyclonal antiserum has been shown to specifically immunoprecipitate cathepsin D from metabolically labeled MCF-7 cells.

Insulin-like growth factor receptor expression and function in fibroblasts from patients with deletion of the distal long arm of chromosome 15.

We have continued our studies on fibroblasts from two patients with deletion of one copy of the distal long arm of chromosome 15. This deletion includes the IGF-I receptor gene in both of our patients. We have measured the level of the IGF-I receptor mRNA in fibroblasts grown under two different conditions, subconfluent cultures in log-phase growth and confluent cultures that were serum-starved. IGF-I receptor mRNA levels were measured by a solution hybridization/RNase protection assay. A riboprobe for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was included in the assay to control for losses during sample handling during the assay. The level of IGF-I receptor mRNA was significantly lower in the newborn patient compared to two age-matched controls under both growth conditions but there was no difference between mRNA levels in the 10 year old patient compared to two age-matched controls.

We previously provided evidence for decreased cell surface expression of the IGF-I receptor in the patients' fibroblasts compared to controls by measuring the amount of  $^{125}\text{I}$ -LR $^3$ IGF-I that was competed by a monoclonal antibody to the IGF-I receptor,  $\alpha$ -IR-3. We attempted to extend these observations by performing Scatchard analysis of LR $^3$ IGF-I binding to fibroblasts from patients and controls. The binding data were analyzed using the program "LIGAND". The binding data for the older patient and controls could not be accommodated to a two site model. The binding data for the newborn patient and controls were consistent with a two site model (presumably a high affinity IGF-I receptor site and a low affinity cell surface binding protein site). Although the number of high affinity binding sites on the patient's fibroblasts was approximately one-half of the level in the control fibroblasts, this difference was not significant because of the large standard error for measurement of receptor number in this analysis. Thus we were unable to extend our earlier results obtained using a different experimental method.

To assess IGF-I receptor function we had earlier examined the stimulation of the nonmetabolizable amino acid, N-methyl-[ $^{14}\text{C}$ ] $\alpha$ -aminoisobutyric acid, uptake in fibroblasts from the patients and controls by a full range of IGF-I concentrations. There were no differences between the patient and control fibroblasts. We have now examined the stimulation of [ $^3\text{H}$ ]thymidine incorporation into DNA by IGF-I as a further assessment of IGF-I receptor function. The response to IGF-I was first optimized by determining concentrations of epidermal growth factor and platelet derived growth factor which by themselves did not cause significant stimulation in the assay but potentiated the response to IGF-I. There was no significant difference between fibroblasts from the patients and controls for ED $_{50}$  for IGF-I. We conclude that although there is evidence for decreased cell surface expression of the IGF-I receptor on the cell surface of fibroblasts of patients with chromosome 15q deletion syndrome, receptor function as assessed by two different bioassays is not impaired.

Signaling by the IGF-I receptor in MG-63 human osteosarcoma cells.

The human osteosarcoma cell line, MG-63, has characteristics which make it an attractive model system in which to study the growth promoting action of IGF-I. MG-63 cells multiply in serum-free medium with IGF-I as the only growth factor, and growth stimulation is mediated by the IGF-I receptor.

Extracellular signal-regulated kinases (ERKs) 1 and 2, also called MAP kinases (denoting either mitogen-activated protein or microtubule-associated protein-2 kinases) have been implicated as being important serine/threonine kinases in the signaling pathway leading to growth and differentiation. ERK1 and ERK2 require phosphorylation on tyrosine and threonine for activation. We asked whether

ERK1 and ERK2 are activated in MG-63 cells by IGF-I. Serum-starved MG-63 cells were treated with IGF-I and after 30 sec, 1, 2, and 3 min, the cell monolayers were lysed with buffer containing 1% Triton X-100 and phosphatase inhibitors. The cell lysates were analyzed by either affinity chromatography on anti-phosphotyrosine-Agarose or by Mono Q-HPLC fractionation. When eluants from the anti-phosphotyrosine-Agarose were immunoblotted with anti-MAP kinase antibody, an IGF-I dependent band of approximately 45 kDa was clearly seen. Two peaks of MAP kinase activity were seen on the Mono Q column. Treatment with IGF-I resulted in an increase in the activity of the first peak. When Mono Q column fractions were TCA precipitated, subjected to gel electrophoresis, and immunoblotted with anti-MAP kinase and anti-phosphotyrosine antibody, the first peak of MAP kinase activity coeluted with ERK1 (44 kDa) and ERK2 (42 kDa) tyrosine phosphorylated protein while the second peak coeluted with ERK1 phosphotyrosine protein. Treatment with IGF-I resulted in an increase in tyrosine-phosphorylated ERK2. We conclude that the increase in MAP kinase activity in the first peak is due to activation of ERK2. Thus in MG-63 cells ERK1 appears to be already activated in serum-starved cells and ERK2 is activated after addition of IGF-I.

#### Publications:

Sklar MM, Thomas CL, Municchi G, Roberts CT, LeRoith D, Kiess W, Nissley SP. Developmental expression of rat insulin-like growth factor-II/mannose 6-phosphate receptor messenger ribonucleic acid. *Endocrinology* 1992;130:3484-91.

Lopaczynski W, Harris S, Nissley P. Insulin-like growth factor-I (IGF-I) dependent phosphorylation of the IGF-I receptor in MG-63 cells. *Regulatory Peptides* 1993; in press.

Nissley P, Kiess W, Sklar M. The developmental expression of the insulin-like factor-II/mannose 6-phosphate receptor. *Molecular Reproduction and Development* 1993; in press.

Nissley P, Lopaczynski W. Insulin-like growth factor receptors and signaling mechanisms. In: Muller EE, Locatelli V, eds. *Growth hormone and somatomedins during lifespan*. Heidelberg: Springer-Verlag 1993; in press.

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

PROJECT NUMBER

Z01 CB 04017-15 MET

PERIOD COVERED

October 1, 1992 through September 30, 1993

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

Biology of the Immune Response

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

David L. Nelson, M.D.	Section Chief	MET, NCI
Jeffrey D. White, M.D.	Clinical Associate	MET, NCI
Glen Bock, M.D.	Special Volunteer	MET, NCI
Harielia Litou, M.D.	Special Volunteer	MET, NCI
S. Bhagovati, M.D.	Expert	MET, NCI
Christine Fredriksen, M.D.	Visiting Fellow	MET, NCI

COOPERATING UNITS (if any)

Children's Hospital, Washington, D.C.

LAB/BRANCH

Metabolism Branch

SECTION

Immunophysiology

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.8	4	1.8 B 100%

CHECK APPROPRIATE BOXES)

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

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

Studies were 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 with its cell membrane receptor (IL-2R) plays a pivotal role in the generation of immune responses. We have identified a soluble form of the IL-2R (sIL-2R) in the serum of normal individuals and found elevated levels of this receptor in a variety of malignancies of the lymphoreticular system. In patients with the Adult T-cell Leukemia (ATL), reductions in serum levels of sIL-2R correlated with responses to chemotherapy. In collaboration with Dr. Thomas Waldmann, this has also been shown in ATL patients receiving IL-2R directed therapies. Elevated serum levels of sIL-2R were also observed in patients with the acquired immune deficiency syndrome (AIDS) and carriers of the Human Immunodeficiency Virus type 1. Thus the measurement of sIL-2R is useful in the management of patients with immunologic activation in vivo. Another T-cell derived lymphokine, interleukin-6 (IL-6) plays a pivotal role in B-cell maturation. We have recently established an IL-6 responsive human tumor cell line which shares many features with the lymphoreticular malignancies occurring in AIDS patients. We have developed two monoclonal antibodies which react with this tumor cell line which do not react with normal resting peripheral blood mononuclear cells. These monoclonal antibodies may be useful in the diagnosis or treatment of AIDS lymphomas. We are current investigating the expression of tyrosine kinase responsible for Bruton's X-linked agammaglobulinemia, BTK, in patients with X-linked agammaglobulinemia and isolated growth hormone deficiency.

## Project Description

### Major Findings:

The cell membrane receptor for the T-cell derived lymphokine, interleukin-2 (IL-2) is a multichain structure consisting of at least two subunits termed the  $\alpha$  (55 kDa) and  $\beta$  (75 kDa) chains of the IL-2 receptor (IL-2R). Using hybridoma-derived monoclonal antibodies to the IL-2R $\alpha$ , we have identified a soluble form of this molecule which is 10 kDa smaller than the cell surface form of IL-2R $\alpha$  and established an Enzyme-Linked ImmunoAssay (ELISA) for the measurement of this molecule in serum.

Elevated levels of soluble IL-2R $\alpha$  were found in diseases associated with human retroviral infections including the Adult T-cell Leukemia (ATL), hairy cell leukemia (HCL), the acquired immune deficiency syndrome (AIDS), and Kawasaki disease. Elevations of soluble IL-2R $\alpha$  were also observed in allograft rejection episodes and exacerbations in autoimmune diseases. Reductions in sIL-2R $\alpha$  correlated with responses to therapy in patients with ATL and HCL. The measurement of sIL-2R $\alpha$  is useful in the diagnosis and management of patients with neoplastic and other inflammatory disorders.

The T-cell derived lymphokine, interleukin-6 (IL-6) plays a pivotal role in B-cell growth and maturation. An IL-6 dependent human tumor cell line has been derived from a patient with intestinal lymphangiectasia, a secondary immunodeficiency disease. Karyotypic abnormalities included t(8;22) (q24;q11) and t(7;14) (q32;q32). Epstein-Barr virus was not detected. Northern analysis revealed a normal 2.4 kb transcript with Myc 1st and 3rd exon probes. Southern analysis with Myc probes localized the translocation breakpoint to the intervening sequence immediately 5' of the 1st Myc exon or within the 5' region of the 1st exon. This is a previously undescribed breakpoint for a t(8;22) (q24;q11) and is of particular interest since it occurred in a tumor which otherwise resembles those of patients with the acquired immunodeficiency syndrome (AIDS). This lymphoid cell line shares many characteristics with the lymphomas occurring in patients with AIDS. Studies are currently underway to use this cell line to develop strategies for the diagnosis and treatment of lymphomas in patients with AIDS.

A deficiency in a cellular protein tyrosine kinase termed BTK has recently been shown to be responsible for conventional or Bruton's X-linked agammaglobulinemia (XLA). Using reverse transcriptase/polymerase chain reaction RT-PCR techniques we are investigating the status of this gene in patients with X-linked agammaglobulinemia and isolated growth hormone deficiency (XLA/GHD). We have also produced a polyclonal antibody recognizing BTK and will be analyzing the expression of BTK and its kinase activity in XLA/GHD. We are cloning full-length cDNAs for BTK and will use Epstein-Barr virus transformed B-cell lines from patients with XLA as an *in vitro* target for retrovirus-based gene therapy prior to attempting peripheral stem cell correction in patients with XLA and XLA/GHD.

### Publications:

Kurman CC, Rubin LA, Nelson DL. Soluble products of immune activation: soluble interleukin-2 receptor (sIL-2R, Tac protein). In: Rose NR, deMacario EC, Fahey JL, Friedman H, Penn GM, eds. Manual of Clinical Laboratory Immunology. Washington, DC: Amer Soc for Microbiol 1992;256-60.

Nelson DL, Kurman CC, Notarangelo LD. The cells and molecules of the immune system. Prospective in *Pediatrics* 1992;85:5-11.

Waldmann TA, Goldman C, Top L, Grant A, Burton J, Bamford R, Roessler E, Horak I, Zaknoen S, Kasten-Sportes C, White J, Nelson D. The interleukin-2 receptor: a target for immunotherapy. In: Gupta S, Waldmann TA, eds. Mechanisms of lymphocyte activation and immune regulation IV: cellular communications. New York: Plenum 1992;57-66.

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

PROJECT NUMBER

Z01 CB 04020-16

PERIOD COVERED

October 1, 1992 through September 30, 1993

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 cancer and AIDS**

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

Jay A. Berzofsky, M.D., Ph.D.	Section Chief	MET, NCI
Richard England, M.D., Ph.D.	Senior Staff Fellow	MET, NCI
Kazutak Kurokohchi, M.D., Ph.D.	Visiting Fellow	MET, NCI
Margaret Marshall, M.D.	Clinical Associate	MET, NCI
Taku Tsukui, M.D., Ph.D.	Visiting Fellow	MET, NCI
Michael Yanuck	HHMI Scholar	MET, NCI

COOPERATING UNITS (if any)

Gene M. Shearer, Ph.D.	Section Chief	EIB, NCI
Richard Hodes, M.D.	Section Chief	EIB, NCI
Melanie Vacchio, Ph.D.	Postdoctoral Fellow	EIB, NCI

LAB/BRANCH

Metabolism Branch (More professional personnel listed on next page)

SECTION

Molecular Immunogenetics and Vaccine Research Section

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

10.5

PROFESSIONAL:

8.5

OTHER:

2 B 100%

CHECK APPROPRIATE BOX(ES)

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

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

We studied the mechanisms by which T cells recognize antigens presented on the surface of cells bound to major histocompatibility (MHC) molecules, the molecular basis of peptide binding to MHC molecules, and the application of these principles to the design of synthetic vaccines for AIDS and cancer. By studying synthetic peptides corresponding to antigenic epitopes, but with amino acid substitutions at different positions, we identified mutations that produced higher affinity for the MHC molecule or for the T-cell receptor. One such peptide from the HIV envelope binds more tightly to a class II MHC molecule and is effective as a vaccine in mice at 10-100-fold lower doses than the natural sequence at eliciting helper T-cells specific for the natural HIV epitope. We also showed that only a few residues on the peptide are critical for positive interaction, but other residues can interfere by adverse interactions, which play a major role in specificity. We found that cytotoxic T lymphocytes (CTL) specific for another HIV peptide from three strains of mice showed degenerate MHC restriction, and also used a limited number of T-cell receptor V-region genes. This same HIV peptide is also presented by both class I and class II MHC molecules to CTL and helper T cells, respectively. We compared the amino acid residues involved in presentation by each, and found a remarkable similarity, that may suggest a similar basis for binding. We used these epitopes to make a synthetic peptide candidate vaccine for HIV. The toxicology and clinical protocols are being written for a human phase I clinical trial. We have also developed a new method of immunizing with peptides without adjuvant, using dendritic cells. We applied this to cancer vaccine development, to show that an endogenously expressed mutant p53 oncoprotein in a tumor cell can serve as a target antigen for CTL, and that such CTL can be elicited by immunization with a synthetic peptide from the mutant p53 sequence. We have also begun determining helper and CTL responses to peptides from human papillomavirus (HPV) oncoproteins E6 and E7, for application to HPV-related cervical cancer. We also found that CTL activity to viruses can be inhibited by Schistosoma infection related to a shift in Th1/Th2 helper balance and mediated by a suppressor cell that we are characterizing. This finding may account for the more rapid progression of HIV disease where these parasites are endemic. We have also found that the immune defect in HIV-infected patients in in vitro T-cell response can be overcome by use of anti-IL-10 antibodies, suggesting a possible approach to therapy.

Cooperating Units:

Peter Nara, DVM	Section Chief	LTCB, NCI
Louis H. Miller, M.D.	Lab Chief	LMR, NIAID
Ronald N. Germain, M.D., Ph.D.	Section Chief	LI, NIAID
David Margulies, M.D., Ph.D.	Senior Investigator	LI, NIAID
Steve Kozlowski, M.D.	Fellow	LI, NIAID
Alan Sher, M.D.	Section Chief	LPD, NIAID
Jeffrey Actor, Ph.D.	Postdoctoral Fellow	LPD, NIAID
Mark Buller, M.D.	Senior Investigator	LVD, NIAID
Bernard Moss, M.D., Ph.D.	Lab Chief	LVD, NIAID
Mario Clerici, M.D.	Visiting Fellow	EIB, NCI
Sanjai Kumar, Ph.D.	Staff Fellow	LMR, NIAID
Stephen Feinstone, M.D.	Senior Investigator	CBER, FDA
Peter Howley, M.D.	Lab Chief	LTVB, NCI
Stephen Hoffman, M.D.	Commander	ID, NMRI ID, NMRI
Walter Weiss, M.D.	Lt. Commander	ID, NMRI
Mark Schiffman, M.D.	Senior Investigator	EEB, DCE, NCI

Project DescriptionMajor 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, malaria and cancer. T cells recognize antigen after it has been proteolytically processed into fragments or unfolded forms which then associate with MHC molecules on another cell, called an antigen-presenting cell by virtue of this function. Almost any cell can present endogenously synthesized antigen with class I MHC molecules, but dendritic cells, macrophages, and B cells specialize in presenting exogenous antigen with class II MHC molecules. Each of these steps can influence which antigenic determinants are seen by T cells.

A major question we have been addressing is the molecular basis for peptide binding to MHC molecules, and the recognition of these peptide-MHC complexes by T-cell receptors, in order to use such information to design improved immunogens and vaccines. One approach we have used is to prepare amino acid variants of an antigenic peptide, each with a single amino acid substitution, and measure the effect of the substitution on T-cell activation and peptide binding to the relevant MHC molecule. By this approach, we hope to determine the role of each amino acid of the peptide in the interactions with T-cell receptor and MHC molecule. In one such study, using myoglobin peptide 102-118, we compared the ability of 42 variant peptides containing single amino acid substitutions to stimulate two different T-cell clones and to compete for binding to the I-A<sup>d</sup> class II MHC molecule, and found that residues interacting with the T-cell receptor were aligned along one face of the peptide, whereas residues interacting the class II MHC molecule were aligned on the other faces. Interestingly, it was the hydrophobic side that interacted with the T-cell receptor. Also, two cases of peptides active in stimulating one T cell at a thousand-fold lower concentration than the native sequence ("heteroclitic peptides") were found. Both were shown to be due to higher affinity of the T-cell receptor for the peptide-MHC complex, rather than a higher affinity of the peptide for the MHC molecule, as is more usually the case. The peptides showed no increase in potency for stimulating another T cell specific for the same determinant, and showed no difference in ability to compete for binding to the class II MHC molecule. One heteroclitic peptide appeared to have a substitution that led to a more favorable interaction, and only one amino acid at that position resulted in heteroclicity. However, the second heteroclitic peptide appeared to have a substitution that removed a deleterious contact, and several smaller amino acids at that position resulted in heteroclicity.

A second study of amino acid substitutions within a peptide focused on a sequence from HIV-1 gp160, called T1, that we had previously shown to stimulate helper T cells specific for the HIV-1 envelope from both mice and humans (see below). This study was carried out in collaboration with the lab of Dr. Ronald Germain. We identified a heteroclitic peptide that had several orders of magnitude higher affinity for the class II MHC molecule than the native sequence. In this case, the substitution appeared to remove an unfavorable negative charge, as replacement of a Glu with either a smaller Ala or an uncharged Gln of the same size, but with an amide instead of a free carboxyl group, resulted in higher affinity binding to the MHC molecule, whereas replacement with an Asp produced no improvement. We conclude from both of these studies that failures of peptides to bind to class II MHC molecules or T-cell receptors often depend more on the presence of residues that interfere with binding than on the absence of residues essential for binding. This explanation of binding (or Ir gene defects) makes sense if the MHC molecule must bind many unrelated peptides. Moreover, it provides a way of developing more potent peptide vaccines, but removing deleterious interactions without affecting specificity. Thus, we have found that we can immunize mice with the heteroclitic HIV peptide analogue with the Glu to Ala substitution at 10 to 100-fold lower doses than required for the natural HIV sequence, and yet still obtain T cells that react well with the natural HIV sequence.

In this same study, we also showed that we could replace all but 4 essential residues with Ala, and still achieve binding to the class II MHC molecule I-E<sup>a</sup>, and antigen presentation (and, indeed, immunogenicity). Thus, for class II as for class I, only a few critical anchor residues are essential for binding and presentation of peptide. We also showed that a single residue on the floor of the peptide binding groove of the MHC molecule was critical for presentation of this HIV helper peptide. I-E<sup>a</sup> (29Val) presented, but I-E<sup>b</sup> (29Glu) did not. However, I-E<sup>b</sup> with residue 29 replaced with Val presented the peptide, whereas, conversely, replacing the Val with Glu at 29 in I-E<sup>a</sup> led to loss of presentation. Again, a limited number of residues are essential for the specificity of binding, both on the MHC molecule and on the peptide.

We have also found that a short, 10-residue peptide from the V3 loop of gp120 of HIV-1 IIIB is presented by 4 distinct class I MHC molecules in the mouse and at least three in the human. Interestingly, in the mouse, the same class I molecules present an apparently unrelated peptide from the C-terminus of gp41, which crossreacts for T-cell recognition. We have not been able to determine the molecular basis for the crossreaction. However, we found that three of the four class I molecules will present the peptide in a degenerate way to T cells of all three MHC types. Thus, these CTL show degenerate MHC restriction, similar to that shown for helper T cells to cytochrome c in the mouse. The T cells which have both properties of crossreactive recognition of the two peptides and degenerate MHC restriction use predominantly one V $\beta$  family T cell receptor, V $\beta$ 8 (and some V $\beta$ 14) as found in a collaborative study with Drs. Richard Hodes and Melanie Vacchio. We also found that the repertoire of T cell receptors is plastic enough that in a mouse strain in which the V $\beta$ 8 receptors are deleted from the genome, it is still possible to generate receptors with this unusual specificity. Thus, if certain receptors are selectively deleted in HIV-infected patients, it may still be possible for the immune system to generate similar specificities using other V-region genes.

The same peptide, P18 of HIV-1 IIIB, is also presented by both class I and class II MHC molecules, as we have shown earlier. We have now examined the molecular basis for binding of this peptide to the two classes of MHC molecule and presentation to T cells. We found that almost the same core region is required for both types of recognition, RGPGRFVTI being required for class I recognition, and IQRGPGRAFVT being required for class II. Furthermore, when we examined peptides substituted at each position in the sequence for binding to class I and class II molecules and for recognition by CD4 and CD8<sup>+</sup> T cells, we found that the residues necessary for binding to the D<sup>d</sup> class I molecule correspond exactly to the newly described motif for peptides eluted from D<sup>d</sup> described by Dave Margulies' lab. Moreover, three of the same residues were also important for binding to the class II



molecule I-A<sup>d</sup>. Similarly, two of the residues that seem to interact with the T-cell receptor in the case of CD8<sup>+</sup> T cells also have the same function for CD4<sup>+</sup> T cells. Thus, there is a remarkable concordance in the molecular basis for binding and presentation of this peptide by class I and class II molecules. Such a comparison has never been done before, and raises questions about possible selective advantage to having a class I and a class II MHC molecule in the same MHC haplotype (H-2<sup>d</sup>) that can present the same peptides.

In the arena of HIV vaccines, we have now made improved synthetic peptide constructs containing the multideterminant cluster helper epitopes and the MN variant of the P18 CTL and neutralizing antibody epitope. The MN variant is more representative of the strains of HIV prevalent in North America and Europe than the IIBB strain used originally. We also truncated the cluster peptide 6 (PCLUS6) helper site by 6 residues at the N terminus to avoid inclusion of a region defined by Hanah Golding as crossreacting with HLA-DR. We have found that this new construct, PCLUS6.1-18MN is as effective an immunogen as the original PCLUS6-18MN in inducing both neutralizing antibodies in multiple strains of mice and in inducing CD8<sup>+</sup> CTL. We have had this peptide and the PCLUS3-18MN peptide made under GMP conditions for a phase I human trial for immunotherapy. For this trial, we have compared several potential adjuvants for their ability to induce both neutralizing antibodies and CTL, since the original complete Freund's adjuvant used for antibodies is not acceptable for use in humans, and since the adjuvant used to date for CTL induction was not Freund's. We compared human grades of incomplete Freund's adjuvant, which has been used in over 900,000 people with little problem, QS21, the non-toxic saponin component of Quil A that retains the adjuvant properties, and alum, since this is the most commonly used adjuvant in human vaccines even though it is not the most potent. We also compared DOTAP, a cationic lipid used for lipofection of DNA into cells, that has been shown to act as an adjuvant for CTL induction. Incomplete Freund's turned out to be the best of these for all responses measured, neutralizing antibodies, CTL, and Th1 helper T-cell responses, as measured by production of IL-2 and not IL-4. Thus, we plan to use this formulation in the planned toxicology studies that are necessary prior to obtaining an IND for the clinical trial.

At the same time, we are starting to develop second generation vaccines taking advantage of the ability described above to enhance antigen presentation by removing side chains that interfere with binding to MHC molecules. These newer constructs are just being tried in mice. We are also carrying out studies using human HLA molecules to determine binding of HIV peptides to these, for further improvements in vaccines. We have found, in collaboration with Drs. Ken Parker and John Coligan, that the same minimal site peptide of HIV-1 gp160 IIBB peptide P18 that binds to the murine class I MHC molecule also binds to HLA-A2, and we are now carrying out studies with HLA-A3, which can also present P18 in functional studies.

We have also developed a new method of immunizing with peptides without the need for adjuvant, to elicit CD8<sup>+</sup> CTL. In the mouse, we can immunize with peptide-pulsed syngeneic irradiated spleen cells to induce CTL. We have found that the dendritic cells are the most active cell in the spleen for this immunization, and purified dendritic cells are much more active than splenic adherent macrophages or B cells. We also found that irradiation is required of the cells, and that this irradiation is not due to a need for inactivation of B cells, but probably has to do with homing of the cells after i.v. injection. Immunization i.v. is much more effective than s.c. or i.p. The CTL immunity lasts at least 6 months after a single immunization. Since dendritic cells are present in human peripheral blood, this should serve as a convenient source of such cells for immunization in humans.

In the area of cancer vaccines, we have shown that we can immunize mice with a synthetic peptide corresponding to a mutant human p53 oncoprotein, containing a point mutation believed responsible for a human lung carcinoma, and elicit CD8<sup>+</sup> CTL that will kill targets incubated with peptide or expressing the mutant p53 endogenously. We have mapped the minimal epitope within this peptide to a sequence

that contains only the point mutation and no mouse-human differences. The point mutation creates a new motif for binding to the K<sup>d</sup> class I MHC molecule, and so creates a neoantigenic determinant where there was none before. The CTL will kill cells transfected with the mutant p53, but not a different mutant human p53, indicating specificity for this site in the endogenous protein. The levels of expression are at the low end of those found in tumors. Thus, we conclude that endogenously synthesized mutant p53 can serve as a target tumor antigen for CTL to kill tumor cells, and that such CTL can be elicited by immunizing with a synthetic peptide. We have also now shown a similar response to a second p53 mutation. We have started immunizing mice to do protection studies, to determine whether peptide immunization will protect against challenge with tumor cells, or will result in rejection of pre-existing tumor. We are also now preparing a clinical protocol to see if such CTL responses can be generated in human cancer patients with p53 mutations, by immunization with synthetic peptides corresponding to their mutation. The p53 genes would be sequenced by Dr. David Carbone (with Dr. John Minna, U. Texas Southwestern in Dallas), and the immunological studies carried out in our lab. The patients, with lung, colon, and breast cancer, would come both from Dallas and from the NCI-Navy Medical Oncology Branch (Drs. Bruce Johnson and Carmen Allegra).

We have also started to study T-cell responses to human papillomavirus oncoproteins E6 and E7, in both mice and humans, as potential targets for prophylactic or immunotherapeutic vaccines. In collaboration with Dr. Richard Houghten, we have prepared overlapping peptides covering both proteins, and Dr. Bernard Moss has prepared recombinant vaccinia viruses expressing these proteins for this project. We have begun to map helper and CTL epitopes in mice, as well as to do protection studies. We have also set up collaborations with Dr. Joe Lucci, an OB-GYN specialist in the group in Dallas, and Dr. Mark Schiffman, of NCI Epidemiology, to study T cell responses in peripheral blood cells of human patients with cervical dysplasia, carcinoma in situ, or invasive cervical carcinoma. In this regard, we are studying both helper and cytotoxic responses, and have already identified several patients with T-cells responsive to peptides. These peptides may be of value for both diagnosis and vaccine development.

In collaboration with Dr. Alan Sher's lab, we have continued to study the effect of schistosome infection on the immune response to viral infection. We have recently found that schistosome infection leads to a shift in the helper T cell response to intercurrent vaccinia viral infection from predominantly Th1 to predominantly Th2, and that this correlates with a loss of virus-specific CTL activity as well as delayed clearance of virus from liver, spleen, and lung. We have now found that the spleen cells from the schistosome-infected mice, which do not display CTL responses to antigens of viruses with which they are infected during the patent stage of parasite infection, actually suppress the CTL responses to virus of spleen cells from non-schistosome-infected mice. The cell responsible for the suppression appears to be a T cell, and is currently being characterized. Because this effect may account for the more rapid progression of HIV infection in countries in which such parasites are prevalent, it is important to work out the mechanism of this interaction and determine how to counteract it.

Finally, in collaboration with Drs. Gene Shearer and Mario Clerici, NCI, we have found that the defect in T-cell proliferative response and IL-2 production to HIV antigens in asymptomatic HIV-seropositive patients can be overcome by anti-IL10 monoclonal antibody in the in vitro culture. This result is consistent with their observation that as HIV infection progresses, the Th1 response declines and the Th2 response increases before both decline. Thus, some of the inhibition of Th1 immunity in HIV infection may be due to Th2 cytokines like IL-10, and may be able to be overcome by anti-IL-10 or other IL-10 antagonists in a therapeutic protocol.

#### Honors and Awards:

McLaughlin Visiting Professorship, University of Texas, 1992  
President, American Society for Clinical Investigation, 1993

Publications:

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Takahashi HY, Nakagawa Y, Takeuchi M, Yokomuro K, Berzofsky JA. Elicitation of CD8<sup>+</sup> class-I-restricted CTLs by immunization with syngeneic irradiated HIV-1 envelope-derived peptide-pulsed splenic dendritic cells. In: Brown F, Chanock RM, Ginsberg HS, Lerner RA, eds. *Vaccines 93*. Cold Spring Harb: Cold Spring Harb Lab, 1993; in press.

Takahashi HY, Nakagawa K, Yokomuro K, Berzofsky JA. Induction of CD8<sup>+</sup>CTL by immunization with syngeneic irradiated HIV-1 envelope derived peptide-pulsed dendritic cells. *Int Immunol* 5 1993; in press.

Takashita T, Kozlowski S, England RD, Brower R, Schneck J, Takahashi H, DeLisi C, Margulies DH, Berzofsky JA. Role of conserved regions of class MHC molecules in the activation of CD8<sup>+</sup>CTL by peptide and purified cell-free class I molecules. *Int Immunol* 5, 1993; in press.

Wasserman GM, Kumar S, Ahlers J, Ramsdell F, Berzofsky JA, Miller LH. An approach to development of specific T lymphocyte lines using preprocessed antigens in murine malaria *Plasmodium vinckei*. *Infect Immun* 1993;61:1958-63.

Weiss WR, Berzofsky JA, Houghten RA, Sedegah M, Hollindale M, Hoffman SL. A T cell clone directed at the circumsporozoite protein which protects mice against both *Plasmodium yoelii* and *Plasmodium berghei*. *J Immunol* 1992;149:2103-09.

Yanuck M, Carbone DP, Pendleton CD, Tsukui T, Winter SF, Minna JD, Berzofsky JA. A mutant p53 tumor suppressor protein is a target for peptide-induced CD8<sup>+</sup>cytotoxic T cells. *Cancer Res*, 1993; in press.

#### Patent Applications:

Berzofsky JA, Yanuck M, Takahashi H, Carbone DP, Minna JD. Novel immunotherapeutic methods and vaccine. Filed March 15, 1993. Application No. 08/031,494.

Berzofsky JA, Ahlers JD, Pendleton CD, Nara P, Shirai M. Composite synthetic peptide construct eliciting neutralizing antibodies and cytotoxic T lymphocytes against HIV. Filed May 14, 1993. Application No. 08/060,988.

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

PROJECT NUMBER

Z01 CB 04024-6

PERIOD COVERED

October 1, 1992 through September 30, 1993

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

**Molecular Analysis of Lymphoid Gene Expression**

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

Louis M. Staudt, M.D., Ph.D.	Senior Staff Fellow	MET, NCI
Alex Dent, Ph.D.	IRTA Fellow	MET, NCI
Chi Ma, Ph.D.	Fogarty Visiting Associate	MET, NCI
Jaya Jagadeesh	Visiting Associate	MET, NCI
Randall Maile	General Fellowship	MET, NCI
Hon-Sum Ko, M.D.	Fogarty Visiting Associate	MET, NCI
Peggy Scherle, Ph.D.	IRTA Fellow	MET, NCI

COOPERATING UNITS (if any)

Jonathan Yewdell, Lab Viral Disease, NIAID

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

7

PROFESSIONAL:

5

OTHER:

2

B 100%

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.)

Dr. Staudt's laboratory focuses on the molecular cloning and characterization of novel lymphoid-restricted genes that regulate the development and function of lymphocytes. Subtractive hybridization cDNA libraries have been used in conjunction with differential screening of cDNA libraries and automated DNA sequencing to isolate genes that are expressed predominantly in B and/or T lymphocytes. One lymphoid-restricted gene, Ly-GDI, encodes a protein bearing striking homology to a regulator of the ras-like G protein, rho. Ly-GDI inhibits the GTP/GDP exchange of rho and becomes phosphorylated during T cell activation. The cloning of Ly-GDI suggests a mechanism by which signal transduction through the ras-like GTP binding proteins can be regulated in a cell type-specific fashion. Another lymphoid-restricted gene, JAW1, encodes a transmembrane protein which, surprisingly, resides in the endoplasmic reticulum. JAW1 has structural similarity to proteins involved in vesicle transport and fusion suggesting that these processes can be regulated in a lymphoid-restricted fashion. Subtracted cDNA libraries are being generated from normal human lymphocytes or human lymphoid malignancies such as Burkitt's lymphoma and chronic lymphocytic leukemia. Rapid and large scale automated DNA sequencing of such libraries has thus far resulted in the identification of over 30 novel lymphoid-restricted genes. The laboratory is currently characterizing in detail several of these novel genes which are predicted to encode nuclear proteins and which may regulate gene expression in a lymphoid-specific fashion.

## Project Description

### Major Findings:

#### Isolation of Lymphoid-restricted cDNAs by Subtractive Hybridization

One novel lymphoid-restricted gene, Ly-GDI, bears striking homology to GDI, an inhibitor of GTP-GDP exchange for the ras-like protein, rho. Ly-GDI is expressed in spleen and thymus but not in other tissues, placing it in contrast with the ras-like GTP binding proteins themselves that are generally expressed in all cell types. Rabbit polyclonal antibodies to Ly-GDI have revealed that it is located diffusely in the cytoplasm. Ly-GDI was demonstrated to form protein-protein interaction with rhoA in vivo by coimmunoprecipitation of the two proteins from lymphocytes. Furthermore, Ly-GDI inhibited the exchange of GTP for GDP on rhoA and thus keeps rhoA in the inactive, GDP-bound state. A role for Ly-GDI in lymphocyte activation was suggested by the demonstration that Ly-GDI becomes phosphorylated within 5 minutes following stimulation of T lymphocytes with phorbol esters. Like Ly-GDI, several recently described regulators of ras-like GTP binding proteins are cell-type restricted. The cloning of Ly-GDI suggests a mechanism by which different cell types can use the same set of ras-like GTP-binding proteins to achieve different intracellular signalling outcomes.

Another lymphoid-restricted gene, JAW1, encodes a protein with an extended coiled-coil domain. Surprisingly, rabbit polyclonal anti-JAW1 antibodies revealed that JAW1 resides in the endoplasmic reticulum (ER) suggesting that the function or structure of the ER differs between cell types. Biochemical studies have shown that JAW1 associates with the ER by an unusual mechanism in which a hydrophobic domain located near its carboxy terminus is inserted into the ER membrane. When the carboxy terminal 60 amino acids of JAW1, including the transmembrane domain, were transferred to a normally cytoplasmic protein, pyruvate kinase, the protein was targeted to the ER. Removal of these same amino acids from JAW1 resulted in its localization to the cytosol. The presence of a transmembrane domain at the extreme carboxy terminus of JAW1 is relatively rare and is shared by only of few other proteins, many of which have been implicated in the transport and/or fusion of intracellular vesicles. Like many of these vesicular trafficking proteins, JAW1 is a type II membrane protein in which the bulk of the protein faces the cytoplasm. Furthermore, many vesicular trafficking proteins, like JAW1, have extensive coiled-coil domains. These considerations lead to our working hypothesis that JAW1 mediates ER vesicle movement and/or fusion in a lymphoid-restricted fashion.

#### Large-scale Sequencing of cDNAs Derived From Normal and Neoplastic Human Lymphocytes

The advent of automated DNA sequencers and robotic workstations for the preparation of DNA sequencing reactions has made feasible the rapid sequencing of large numbers of cDNAs and thus the ability to generate a cDNA sequence profile of a given cell type. We have applied this approach to our subtractive cDNA libraries and have identified more than 30 novel lymphoid-restricted genes. A variety of genes have been identified which are predicted to encode nuclear proteins, either because they show homology to transcription factors or because they have nuclear localization signals. Several other lymphoid-restricted genes show homology to genes involved in signal transduction. Most of the novel lymphoid-restricted genes bear no obvious sequence relationship to previously cloned genes. Our current work on these novel genes includes the combined use of biochemical and genetic approaches to elucidate their role in lymphocyte development and function.

Publications:

Scherle P, Behrens T, Staudt LM. LyGDI, a GDP dissociation inhibitor of the rhoA GTP-binding protein, is expressed preferentially in lymphocytes. Proc Natl Acad Sci USA 1993, in press.





SUMMARY REPORT  
EXPERIMENTAL IMMUNOLOGY BRANCH  
October 1992 - September 1993

The Experimental Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis in the following areas: 1) lymphocyte differentiation and regulation; 2) cell biology of immune responses; 3) signal transduction; 4) structure, regulation and function of genes involved in immune responses; 5) lymphocyte effector function, 6) developmental biology; 7) transplantation biology; 8) tumor immunology; and 9) 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 intra-thymic differentiation of functionally and phenotypically distinct T cell subsets as well as their interaction with thymic epithelium (9273). Studies on thymocytes from genetically defective scid mice have suggested that Tcr<sup>+</sup> cells play a critical role in promoting the entry of thymocytes into the CD4/CD8 differentiation pathway as well as in promoting the maturation and organization of thymic medullary epithelium. The role of TCR expression by the differentiating thymocytes themselves was studied using scid mice possessing an already rearranged TCRV $\beta$  transgene which allows development of mature T cells in these mice. These studies showed that T cells at different stages of development in these mice expressed structurally distinct surface TCR complexes, and that the developmental stage reached by individual T cells is related to the structural nature of TCR complexes expressed by those cells. These results are important to our understanding of T cell differentiation because they suggest that successful T cell differentiation normally requires surface expression of fully assembled TCR complexes.

Dr. A Singer's laboratory has also studied early thymocyte differentiation by *in vivo* and *in vitro* analyses of requirements for the transition of precursor thymocytes of the CD4<sup>-</sup>CD8<sup>10</sup> phenotype into CD4<sup>+</sup>CD8<sup>+</sup> double positive thymocytes (9273). It was found that this differentiation step could be inhibited *in vitro* by TCR crosslinking or by an activator of protein kinase C (PKC), phorbol myristate acetate (PMA). TCR-mediated inhibition of differentiation: 1) was mediated by elimination of messenger RNA's encoding the co-receptor molecules CD4 and CD8 and the recombination activating genes (RAG) 1 and 2; 2) required protein synthesis; and 3) was developmentally regulated. These studies identify a post-transcriptional mechanism that is influenced by TCR signals, and that regulates early thymocyte development.

The non-TCR mediated requirements for progression of developing thymocytes along the CD4/CD8 developmental pathway were also analyzed by Dr. A Singer's laboratory (9273). It was found that progression through one cell cycle is necessary for differentiation of CD4<sup>-</sup>CD8<sup>10</sup> precursor thymocytes in double positive thymocytes, and this progression is specifically regulated by interaction with cortical thymic epithelial cells (TEC). The regulatory ligands expressed by cortical TEC was identified as transforming growth

factor  $\beta 1$  (TGF- $\beta 1$ ) and TGF- $\beta 2$ . These studies identify a novel regulatory mechanism that acts on developing precursor thymocytes independently of TCR, and that is mediated by cortical TEC.

Because *in vitro* antibody-mediated TCR signals could inhibit the differentiation of CD4<sup>+</sup>CD8<sup>10</sup> precursor thymocytes, Dr. A. Singer's laboratory has also investigated the possibility that antigen-mediated TCR signaling could affect negative selection in precursor thymocytes (9273). It was found that in a negatively selecting male thymus, CD4<sup>+</sup>CD8<sup>10</sup> precursor thymocytes that express a transgenic TCR to male antigen are developmentally arrested as a consequence of antigen encounter and fail to become CD4<sup>+</sup>CD8<sup>+</sup>. The *in vivo* differentiation of the same thymocytes in a female thymus progressed normally, but *in vitro* differentiation of female precursor thymocytes could be inhibited by male antigen-presenting cells expressing the appropriate MHC-restriction element. These studies demonstrate that negative selection of the T cell receptor repertoire can occur before the CD4<sup>+</sup>CD8<sup>+</sup> stage of thymocyte differentiation.

The consequences of *in vivo* TCR-mediated negative selection were also examined by Dr. A. Singer's laboratory by analysis of intracellular calcium concentrations (9268). It was found that among thymocytes expressing a transgenic TCR of defined specificity, a large number had elevated intracellular calcium concentrations, but only when resident in a negatively selecting thymus in which their self ligand was expressed. Thus, developing thymocytes are stimulated by endogenous ligands *in vivo* to mobilize intracellular calcium, and increased intracellular calcium concentrations may reflect the consequences of intrathymic signaling associated with thymic negative selection.

The process of negative selection, by which potentially self-reactive T cells are deleted during development, has also been analyzed in the laboratory of Dr. Richard Hodes (9265). Strain-specific deletions in multiple T cell receptor (TCR) V $\beta$  products 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 the available TCR V $\beta$  repertoire. The self determinants that function as ligands for V $\beta$ -specific T cell deletions were shown generally to represent the products of non-MHC-encoded genes in association with MHC gene products. Mapping of the non-MHC-encoded genes contributing to V $\beta$ -specific deleting ligands has in each case identified an endogenous mouse mammary tumor (MMTV) provirus associated with deletion.

The influences of exogenous retroviruses on the T cell repertoire were examined (9265). A defective murine leukemia virus which causes a mouse acquired immune deficiency syndrome (MAIDS) induced superantigen-like T cell activation *in vitro*. *In vivo*, this virus selectively activated and expanded CD4<sup>+</sup> T cells expressing V $\beta 5$ , followed later in the course of infection by widespread immune deficiency in all T cells. These findings indicate the effect of V $\beta$ -specific, superantigen-like stimulation *in vivo* and *in vitro* in response to MAIDS retrovirus.

The biology of the inter-relationship between milk-borne MMTV and the T cell receptor (TCR) repertoire was analyzed (9265). A previously uncharacterized tumorigenic milk-borne virus in BALB/c mice (the BALB/cV virus) was found to induce deletion of T cells expressing TCR V $\beta 2$  in developing mice. This

effect was MHC-dependent. This finding suggests that expression of superantigenic capacity is an essential characteristic of infectious MMTV. The role of superantigenicity in MMTV infection was directly analyzed by testing the influence of major histocompatibility complex (MHC) class II expression on susceptibility to MMTV infection. The milk-borne C3H MMTV induced V $\beta$ 14 deletion only in strains of mice bearing natural or transgenic I-E class II MHC product. Moreover, susceptibility to milk-borne virus, as determined by assays of viral pp28 or LTR mRNA, was also dependent upon I-E expression. These findings indicate that viral infection is dependent upon superantigenic stimulation of host lymphoid cells.

Although V $\beta$ -specific superantigenic effects are a useful model for the study of TCR selection, selection may more commonly be on the basis of receptor specificity determined by multiple TCR  $\alpha$  and  $\beta$  chain components (9265). Analysis of the expression of specific TCR V $\alpha$ /V $\beta$  pairs has indicated that V $\alpha$ /V $\beta$  pairing is non-random, and that strain-specific differences exist in patterns of V $\alpha$ /V $\beta$  expression, providing a new approach to the study of repertoire selection. Additional evidence for the importance of V $\alpha$ /V $\beta$  combinatorial specificity was observed in the response to the endogenous superantigen Mls<sup>a</sup> (mtv-7). When Mls<sup>a</sup>-specific T cells were selected by *in vitro* stimulation, it was found that V $\alpha$  expression, in addition to the dominant influence of V $\beta$  expression, plays a role in T cell specificity for endogenous mtv superantigen.

Dr Stephen Shaw's laboratory has been systematically analyzing heterogeneity among subsets of human T cells and the functional capacities of those subsets (9257). The concept that adhesion molecules often mark T cell subsets has been confirmed and extended by many aspects of the laboratory's studies this year. The laboratory has continued studies of the T cell integrins which mediate strong adhesion to endothelium. Results of this work have confirmed and extended the concepts that: 1) Naive cells are relatively homogenous phenotypically and express a relatively low uniform level of integrins; 2) Memory cells are very heterogeneous in phenotype, and memory cell subsets have specialized adhesion phenotypes. Flow cytometry and 2D-gel analysis identifies predominantly  $\alpha$ 6 $\beta$ 1,  $\alpha$ 4 $\beta$ 1,  $\alpha$ 4 $\beta$ 7, and  $\alpha$ 5 $\beta$ 1 on T cells. The most complex and informative patterns are observed with immunoprecipitations using  $\alpha$ 4, which reveals: 1) intact  $\alpha$ -chain ( $\alpha$ 140) and its cleaved fragments ( $\alpha$ 80 and  $\alpha$ 70); and 2) both  $\beta$ 1 and  $\beta$ 7. Consistently, there is a greater relative abundance of  $\beta$ 7 (relative to  $\beta$ 1) in naive cells, suggesting a relatively greater contribution of  $\beta$ 7 to the adhesive potential of naive cells.

The Cell Mediated Immunity Section, under Dr. Gene Shearer, is investigating human T helper cell (TH) function in: a) asymptomatic HIV-infected (HIV+) individuals (9267); b) HIV-exposed individuals who exhibit no evidence of infection (9267); c) patients with systemic lupus erythematosus (SLE) (9282); and d) cancer patients (9402). It was found that both HIV+ individuals and SLE patients exhibit a spectrum of TH functional defects which are predictive for disease progression and are associated with changes in the profiles of immunoregulatory cytokine production, including interleukins 2, 4, 10, and 12, as well as interferon- $\gamma$ . A significant number of HIV-exposed, seronegative individuals from every known risk group was found to exhibit *in vitro* TH function to synthetic peptides of HIV gp120. Studies in these at-risk groups and newborn infants of HIV+ mothers suggest that HIV-specific TH function is protective against HIV infection and/or progression to AIDS.

## 2. CELL BIOLOGY OF IMMUNE RESPONSES

The expression and function of cell adhesion molecules by B cells was analyzed by Dr. Hodes' laboratory (9266). IL5 induces B cell proliferation and immunoglobulin (Ig) secretion and results in appearance of a phenotypically novel B cell population which expresses high densities of CD44 and low densities of B220 (CD45) and Ia. This B cell subpopulation mediates nearly all of the proliferative and Ig secretory activity of IL5-activated B cells. In addition, the CD44 expressed by these cells mediates binding to the extracellular matrix material hyaluronic acid (HA), indicating a potential role for CD44 in regulating trafficking of activated B cells in vivo. The CD44 expressed on IL5-stimulated B cells migrates with a lower molecular weight than does CD44 expressed by control B cells, reflecting differential glycosylation. No differences in CD44 mRNA isoforms were apparent by PCR analysis. Other B cell activating stimuli, such as LPS, do not induce CD44-dependent HA-binding activity. However, LPS-activated B cells demonstrate CD44-dependent HA binding rapidly after exposure to a unique CD44-specific mAb, suggesting that distinct functional states of the CD44 molecule exist, perhaps reflecting differences in conformation or cytoskeletal association.

T cells at various stages of activation and differentiation are known to express different isoforms of cell surface CD45, reflecting in part the differential splicing of several variable exons. In contrast, B cells have generally been characterized as expressing a uniformly high molecular weight isoform of CD45. Analysis of resting and activated B cells demonstrated that activation-specific changes are induced in the expression of serologically detected CD45 epitopes (9266). These changes can be correlated with changes detected by immunoprecipitation. In addition, a polymerase chain reaction (PCR) analysis of CD45 mRNA expression indicates that unique changes in variable exon splicing are induced by specific B cell activation stimuli.

In order to identify previously uncharacterized activation molecules expressed on lymphocytes, a series of mAb was generated by immunizing rats with activated mouse B cells. One of these mAb (GL7) reacts with a subpopulation of activated B cells, as well as with activated T cells (9266). GL7 precipitates what appears to be a previously undescribed 29-31 KDa molecule from activated B cells. Another mAb generated in this fashion (G11) reacts with activated B but not T cells. G11 inhibits responses of CD4<sup>+</sup> T cells to activated B cells, suggesting that the target of G11 may represent a costimulatory molecule for T cell activation.

Immunoglobulin (Ig) secretion was analyzed in a model system employing Ig  $\mu/k$  transgenic mice (9266). During characterization of Ig  $\mu/k$  transgenic mice, it was noted that a high proportion of serum Ig molecules of endogenous (non-transgenic) origin expressed the transgene idiotype. This observation could have resulted from the existence of mixed isotype Ig molecules, from extensive class switching by trans-rearrangement, or from a "network" influence on Ig expression. Analysis by ELISA, immunoabsorption, and gel filtration demonstrated that transgenic  $\mu$  chains associate in chimeric Ig molecules with endogenous  $\mu$  or  $\alpha$  chains produced by the same cell.

Dr. Shaw's laboratory has made significant progress this year in their continuing studies of T cell adhesion to endothelium (9257). Results of

these studies have strengthened the model that binding occurs via a cascade consisting of at least 3 steps: tether, trigger, and strong adhesion. The triggering step for T cells has previously been the least well defined. Dr. Shaw's laboratory has discovered two "pro-adhesive" cytokines which are potential physiologic triggers: MIP-1 $\beta$  and hepatocyte growth factor (HGF). It was found that MIP-1 $\beta$ : 1) induces T cell adhesion to the endothelial ligand VCAM-1; 2) can be functionally retained on proteoglycan to mediate its adhesion-induction; 3) is found at the endothelial surface in tonsil and lymph node; and 4) is chemotactic for T cells. Although hepatocyte growth factor (HGF) is structurally distinct from MIP-1 $\beta$ , and previously had no known immunologic relevance, it was found that HGF is functional similar to MIP-1 $\beta$  in that HGF is strongly chemotactic for T cells and also induces T cell adhesion. In addition, immunohistologic studies identify HGF on endothelium at sites such as inflamed liver. It has been proposed that these factors are retained at the endothelial surface by binding to proteoglycan. A broad physiologic model has been developed which proposes that such pro-adhesive cytokines are delivered to endothelium by a specialized fibroblastic reticular conduit system.

### 3. SIGNAL TRANSDUCTION

The role of "second messengers" mediating activation of T cells through the TCR/CD3 complex was analyzed in cloned T cell populations by Dr. Hodes (9281). A cloned T cell population that was maintained by repeated stimulation in vitro with IL2 alone was capable of responding to subsequent stimulation with anti-CD3 antibody by proliferating and by strong phosphatidyl inositol (PI) hydrolysis and increased intracellular calcium concentration. In contrast, the same cloned line maintained by stimulation with specific antigen and antigen-presenting cells responded to anti-CD3 stimulation by proliferating, but without measurable PI hydrolysis or calcium response. The ability to transduce a TCR-mediated signal through the PLC pathway in cloned T cells is therefore influenced by prior stimulation through the TCR.

Signal transduction pathways induced by endogenous superantigen stimulation of T cells were analyzed with both cloned and heterogeneous responding T cells (9281). It was found that both PI hydrolysis and increased  $[Ca^{++}]_i$  were induced by Mls<sup>a</sup> (mtv-7) superantigen-bearing APC. Using TCR transgenic mice it was further demonstrated that in mice expressing Mls<sup>a</sup> as a self antigen, no Mls<sup>a</sup>-specific response was induced in peripheral T cells; in contrast thymocytes did respond to self Mls<sup>a</sup> by conjugate formation and increased  $[Ca^{++}]_i$ , demonstrating that immature thymocytes, prior to negative selection, respond specifically to self superantigen.

Receptor-mediated activation was analyzed in T and B lymphocytes from normal mice and from mice infected with the MAIDS-inducing defective murine leukemia virus (9281). Several weeks after viral infection, the proliferative responses of T and B cells to cross-linking of TCR and sIg respectively were significantly reduced despite the expression of normal surface levels of these receptors by most T and B cells. To analyze early signalling events in these cells,  $[Ca^{++}]_i$  was measured in response to surface receptor cross-linking. The  $[Ca^{++}]_i$  responses of both T and B cells from MAIDS-infected mice were decreased. B cell responses to sIg cross-linking were further analyzed by examining protein tyrosine phosphorylation induced by sIg cross-linking. It was found that after virus infection, there was a progressive loss of selected tyrosine phosphorylation events

with conservation of other events. The response defect in B cells from MAIDS mice is thus reflected in selected alterations of tyrosine phosphorylation in response to sIg signaling.

The molecular basis for low antigen receptor expression in developing CD4<sup>+</sup>CD8<sup>+</sup> thymocytes has been studied in Dr. Alfred Singer's laboratory (9268). Their studies revealed that T cell receptor (TCR) expression and function in developing murine thymocytes is actively regulated by CD4-mediated signals generated by the interaction of CD4 with Ia<sup>+</sup> thymic epithelium (9268). They found that CD4 molecules on the surface of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes are engaged *in situ* by Ia<sup>+</sup> thymic epithelium and transduce intracellular signals that result in: (i) low TCR expression, (ii) tyrosine phosphorylation of TCR-zeta chains, and (iii) marginal signaling ability of TCR to flux intracellular calcium upon TCR crosslinking. Dr. Singer's laboratory found that release from intra-thymically generated inhibitory CD4 signals results in increased TCR expression, dephosphorylation of TCR-zeta chains, and improved TCR signaling. Further, Dr. Singer's laboratory has found that the molecular basis for low TCR expression in developing CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is a high rate of degradation of newly synthesized TCR components, and that intrathymically generated CD4 signals mediated by the tyrosine kinase p56 lck regulate the TCR degradation rate in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Further, p56 lck was shown to be preferentially associated with CD4, rather than CD8 in immature thymocytes, a finding which correlated with the competence of CD4, but not CD8, to activate p56 lck in thymocytes. These studies demonstrate a novel function for an intracellular tyrosine kinase in the regulation of TCR distribution and expression in immature thymocytes.

Because lck was found to regulate TCR expression in immature thymocytes, Dr. Singer's laboratory also investigated the possibility that the membrane bound protein tyrosine phosphatase CD45, known to regulate lck activity, might also play a role in thymocyte differentiation (9268). Using both *in vivo* and *in vitro* treatments, it was found that antibody engagement of CD45 on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes: (i) enhanced lck tyrosine kinase activity; (ii) inhibited TCR expression; and (iii) inhibited differentiation of immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes into mature single positive T cells. These studies demonstrate that the ability of immature thymocytes to undergo positive selection can be regulated by CD45 and suggest a potentially important regulatory role for intrathymic ligands of CD45.

#### 4. STRUCTURE, REGULATION AND FUNCTION OF GENES AND PROTEINS INVOLVED IN IMMUNE RESPONSES

The laboratory of Dr. Dinah Singer continues to characterize the molecular mechanisms regulating MHC class I gene expression (9270). Studies from this laboratory have defined two broad categories of regulatory mechanisms: those governing homeostatic, tissue-specific patterns of expression and those governing the dynamic modulation of class I genes. Research in both areas has been pursued. It has been demonstrated that homeostatic levels of class I gene expression are established and maintained by a complex regulatory system consisting of overlapping silencer and enhancer activities. Levels of class I are determined by the equilibrium between these activities. Characterization of the regulatory DNA sequence elements has been completed, and studies are now directed toward the characterization of the cognate DNA binding factors.

Studies of the dynamic regulation of class I have demonstrated that class I gene expression is cyclically regulated in response to hormonal stimulation (9400). It has been shown that transcription of class I genes is repressed in thyrocytes in response to thyroid stimulating hormone. The molecular mechanisms regulating this repression, both the responsive DNA sequence elements and the trans acting factors, have been investigated. These observations led to the hypothesis that modulation of class I levels is necessary to maintain tolerance in endocrine tissues and that failure to properly regulate class I levels could lead to autoimmune responses. Consistent with this model, it has now been demonstrated that the absence of class I expression confers resistance to certain experimental autoimmune diseases *in vivo*.

Class I molecules are the major receptors for viral peptides and serve as targets for specific cytotoxic T lymphocytes. Many viruses are known to repress class I expression. The effect of HIV on class I gene expression was investigated by Dr. Dinah Singer's laboratory (9401). It was found that HIV was able to decrease class I promoter activity by up to 12-fold. Repression was mediated specifically by the HIV Tat protein consisting of two coding exons; Tat derived from a single coding exon did not repress. These studies define an activity for two-exon Tat distinct from that of one-exon Tat. They further raise the possibility that during persistent infection, HIV infected cells express reduced levels of class I providing a mechanism whereby they remain hidden from the immune system.

Dr. Segal's group has made a single-chain Fv (sFv) genetic construct from the T cell receptor of the 2B4 murine T cell hybridoma. The protein has been expressed in bacteria and refolded, to produce sFv protein in 10-100 mg amounts (9289). The resultant protein is monomeric in aqueous solution and contains three epitopes that are present on the parental TCR for which Mabs are available to the laboratory. This recombinant TCR-sFv appears to have biological activity in that it specifically inhibits the presentation of antigen (cytochrome C peptide bound to I-E<sup>K</sup>) to 2B4 cells, and blocks stimulation of T cell hybridomas by appropriate superantigens. Various fusion proteins that have improved solubility and folding properties compared to the original construct have also been produced. These data show that isolated variable domains of the TCR can form a well defined structure with immunological and biological properties similar to those of the native TCR. This protein will be used to study the specificity and structure of the T cell receptor binding site.

## 5. LYMPHOCYTE EFFECTOR FUNCTION

In studies on the mechanism of lymphocyte-mediated cytotoxicity, Dr. Henkart's laboratory has extended the granule exocytosis model to include a role for granzymes (serine proteases in granules) in triggering "apoptotic" damage to target cells (9251). The approach was transfection of a mast cell tumor line with combinations of the lymphocyte granule components cytolysin and granzyme A. Transfectants expressing only the membrane pore-forming cytolysin gave a modest IgE-dependent cytotoxicity without target DNA breakdown, while transfectants expressing both cytolysin and granzyme A gave a potent tumor target killing with accompanying DNA breakdown. In further support of a role for granzymes in target cell damage, it was found that CTL target cells loaded with the protease inhibitor aprotinin were resistant to the lethal hit. It was furthermore shown that injection of several different, well characterized, proteases into tumor cells triggered a rapid

cell death, usually, but not always, accompanied by classical apoptotic features. These results indicate that cells have an internal death pathway responsive to internal proteolysis. These data further support the granule exocytosis model for lymphocyte-mediated cytotoxicity.

Dr. Henkart's laboratory has been studying the mechanism of programmed cell death in lymphocytes (9263). Using protease inhibitors, particularly those directed towards the calcium-activated cysteine protease calpain, this group has identified a cell death pathway involved in the antigen-induced death of mature T lymphocytes. Calpain inhibitors block the TCR-triggered death of T hybridoma cells, activated peripheral T cells, and blood T cells from HIV<sup>+</sup> donors. The pathway identified does not operate for steroid-induced programmed cell death of any cells tested, nor for TCR-triggered death of immature thymocytes. In the T hybridoma cells, TCR-induced IL-2 secretion is not blocked by these inhibitors. Calpain inhibitors partially restore defective T helper proliferative responses of cells from HIV<sup>+</sup> donors. These findings have led to the proposal that calpain inhibitors be used for therapy in HIV infection.

By using bispecific antibodies that have dual specificity for both CD44 and a target cell antigen, Dr. Segal's laboratory has shown that human peripheral blood lymphocytes contain a subset of cells that can be stimulated to lyse target cells through CD44 (9254). Although the precise subset of cells mediating this lysis has not been identified, the activity has been shown to reside in the low buoyant density fraction, most likely in the CD56<sup>+</sup> subset of PBL. The activity arises after 24-48 hours of stimulation with IL-2, but has not been seen in cells stimulated for several days with anti-CD3 plus IL-2; even though these latter cells express high amounts of CD44 and function as targetable killer cells through CD3. These studies show that a prominent adhesion molecule, CD44, can, when engaged, stimulate a fraction of the cells that express it to mediate lysis. They also raise the questions of how CD44 on these cells differs from that on other cytotoxic cells, and what the biological function of this subset of cytotoxic cells is.

## 6. DEVELOPMENTAL BIOLOGY

Dr. Kuehn's laboratory carries out retroviral insertional mutagenesis to identify genes that have important roles during mouse embryonic development. Mouse embryonic stem (ES) cells are used to import into the mouse germ line large numbers on independent proviral insertions, each of which has the potential to induce an insertional mutation. Previous studies used a single retroviral vector to infect ES cells. Due to difficulties in resolving large numbers of proviral bands using Southern blotting, insertional mutagenesis studies are now being done using a multiplex approach (9298). Six vectors constructed by inclusion of different fragments of phage lambda DNA have been used to infect ES cells. In the cells, and transgenic mice derived from them, the number of proviruses of a particular type is low, but the overall copy number is high. Using 6 different probes in series, the inheritance patterns of all 120 proviral insertions can be followed in the transgenic strains. The laboratory has also developed more efficient methods for producing embryos for ES cell injection.

From the previous studies of mice derived from ES cells multiply infected with a single retroviral vector, 5 proviruses that disrupt embryonic development when homozygous have been identified. These presumed recessive



mutations and the genes they identify are the subjects of ongoing studies in Dr. Kuehn's laboratory (9297). The insertion sites have been cloned and are in the process of being genetically mapped. Searches are underway to locate transcribed regions that may represent part(s), or all, of each of the mutated genes. In addition to these molecular studies, the mutant phenotypes are being analyzed histologically.

For one of these mutations, the molecular and phenotypic studies by Dr. Kuehn's laboratory are well advanced (9299). The phenotype has been extensively characterized by histological studies on embryos at all stages of early post-implantation development. Mutant embryos do not form any mesoderm and fail to undergo the first steps of gastrulation. In addition, the embryonic and extra-embryonic lineages hyperproliferate before undergoing degeneration. The gene identified by this mutation has now been isolated and shown to be a new member of the TGF-beta superfamily of secreted growth factors. It is expressed in normal embryos at a time consistent with a role in mesoderm induction. In addition, it is expressed later in an embryonic region called the "node" which is known to play a critical role in the further induction and patterning of mesodermal structures in amniotes.

#### 7. TRANSPLANTATION BIOLOGY

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL) play a significant role in mediating allogeneic marrow graft rejection. In a murine model system studied in Dr. Gress' lab (9287), 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. Cells with a specific type of suppressor activity, termed veto cells, which might suppress host rejection responses, have been reported to be present in marrow. Veto cells suppress those precursor CTL with specificity for antigens expressed on the surface of the veto cells. It was found that the incubation of T cell depleted marrow with IL-2 significantly increased veto activity as assessed by *in vitro* assays and also enhanced engraftment of MHC-mismatched, T cell depleted marrow *in vivo*. Studies showed an inhibition of veto activity by antisera with specificity for cytolytic granules, indicating that lysis of precursor CTL might be the mechanism for the suppression of CTL responses by IL-2 enhanced veto cells. Additional experiments with transgenic mice have demonstrated that clonal deletion, rather than clonal anergy, is in fact the mechanism by which veto cells mediate suppression of the CTL response, and that such clonal deletion involves participation by precursor CTL as well as by veto cells. In further studies of engraftment of T cell depleted allogeneic marrow, host mice were treated with anti-CD3 monoclonal antibody. The injection of anti-CD3 monoclonal antibody with the donor marrow resulted in extensive allogeneic chimerism. Incubation of T cell depleted allogeneic marrow with the supernatant of spleen cells incubated with anti-CD3 antibody *in vitro* also resulted in enhancement of engraftment in the presence of, but not in the absence of, host T cell suppression. Therefore, the enhancement of marrow engraftment by *in vivo* administration of anti-CD3 monoclonal antibody is due to both suppression of host T cell function and the presence of growth factors. Additional studies indicate that the differential ability of donor versus host marrow cells to respond to growth factors contributes to available "space", which in turn contributes to donor marrow engraftment or rejection.

The elimination of cells expressing T cell surface markers from marrow is of interest both in allogeneic and autologous marrow transplantation -- as a means of preventing graft versus host disease in allogeneic marrow transplantation and as a means of eliminating or purging malignant cells expressing T cell surface markers from marrow in treating T cell neoplasms by autologous marrow transplantation. Dr. Gress' laboratory has developed approaches for depleting normal and malignant T cell marrow populations by using elutriation and deriving monoclonal antibodies specific for cell surface molecules unique to T cells (9288). These approaches have been used to develop clinical protocols to assess the feasibility of utilizing allogeneic HLA-mismatched, T cell depleted allogeneic marrow and autologous marrow purged of malignant T cells in the treatment of aggressive hematolymphopietic malignancies. The generation of T cell populations following T cell depleted marrow transplantation has been investigated. In murine studies, three T cell progenitor pools have been identified which contribute to final T cell repopulation following marrow transplantation. Interestingly, interregulation exists among these progenitor pools which determines the extent to which each pool contributes to the final reconstituted T cell population. The functional capacities of regenerated T cell populations following T cell depleted marrow transplantation is also of interest. The human T helper cell response to xenogeneic MHC encoded antigens expressed by stimulating murine cell populations has been studied and found to be of special use in the assessment of human T helper cell function in that this primary response requires reprocessing of the stimulating murine antigens and presentation in association with human Class II gene products. These results were consistent with an Ia-dependent recognition of processed murine antigen by human T cells which represents an approach for assessing human T helper cell function and MHC restriction in a primary T cell response. The requirement for reprocessing of murine antigen and presentation by responder-type cells (rather than murine stimulator cells) was found to be due in part to a lack of murine antigen presenting cell activation. Such activation could be accomplished by the cytokine GM-CSF which resulted in critical upregulation of B7 cells in the APC population. GM-CSF, in turn, was produced by T cells upon stimulation with antigen and costimulation with either CD54 or CD58. These findings define a minimally sufficient pathway of T cell-APC interaction in the production of IL-2.

Dr. Gene Shearer's laboratory is investigating the mechanisms of human solid organ allograft rejection (9264). It was found that renal and cardiac allograft rejection is mediated only by recipient anti-donor CD4+ T helper cell responses that are activated by alloantigen, processed and presented on recipient antigen-presenting cells (APC). In contrast, liver allografts appear to be rejected both by processed alloantigens and alloantigens presented directly on donor APC. The rejection of renal allografts could be predicted by an *in vitro* T cell test developed by the laboratory. This test was predictive for long-term (3-year) survival of functioning kidney allografts.

## 8. TUMOR IMMUNOLOGY

A mouse model for retargeting the immune system against syngeneic mammary tumors has been studied in the laboratories of Drs. Segal and Wunderlich (9250,9254). The model utilizes tumors, both primary and passaged, induced by the vertically transmitted mammary tumor virus. The specificities of

cytotoxic T lymphocytes from normal donors have been retargeted with bispecific antibodies, so that the T cells react against a virus-related envelope protein selectively expressed on the surface of the tumor cells. One bispecific antibody was prepared by chemically crosslinking anti-CD3 and anti-gp52 monoclonal antibodies, and a second was prepared by collaborators at Protein Design Labs, by genetically engineering F(ab')<sub>2</sub> molecules using fos and jun leucine zippers to form the bispecific reagent. It was found that retargeted cytotoxic mouse splenocytes lyse both passaged and freshly explanted primary tumor cells *in vitro*, and that they block the growth of syngeneic tumor cells in subcutaneous tumor neutralization (Winn) assays. In order to mediate these activities, the effector cells needed to be preactivated, and the bispecific antibodies needed to be crosslinked rather than physically mixed.

In other studies, designed to improve targeting reagents, Dr. Segal's laboratory has genetically engineered sFv fusion proteins from anti-DNP and anti-transferrin receptor antibodies, that contain myc-peptide tags at their C-terminal ends (9289). In conjunction with an anti-CD3 x anti-myc bispecific antibody, these sFv proteins induced T cells to lyse appropriate target cells. These results show that, in principle, sFv proteins can be used in redirected lysis, and may provide a way in which a cocktail of sFv constructs could target tumor cells for destruction. In further studies of sFv proteins, it was found that mammalian COS cells produce and secrete active sFv antibodies. Three different sFvs were produced, but were refolded and processed at different rates. These studies show that the mammalian protein folding machinery successfully folds and secretes sFv proteins, and lays the groundwork for the production of a true single chain bispecific antibody.

## 9. FLOW CYTOMETRY

The EIB flow cytometry laboratory (9255) continues to support multiple investigations which involve quantitative, single cell, multiparameter immunofluorescence analysis of cells prepared from a variety of tissues and species, as well as a spectrum of *in vitro* cultured cells (9268, 9273, 9265, 9257, 9266, 9281, 9275, 9287, 9288).

During the past year, the EIB flow cytometry laboratory (9255) has fully implemented use of new flow cytometry instrumentation for research support. Modifications have been designed and implemented to enhance throughput, functionality and flexibility of flow cytometry instrumentation and of associated computer hardware and software.

Dr. Shaw has organized a component of the 5th International Workshop on Leukocyte Differentiation Antigens. This workshop is part of a series of international collaborative undertakings to systematically enumerate and characterize the cell surface molecules relevant to the human immune system. The section organized by Dr. Shaw involves 30 laboratories which are using over 400 particularly informative antibodies to semi-quantitatively measure the expression of most previously identified cell surface molecules on cells of multiple lineages using flow cytometry. Dr. Shaw is coordinating all data analysis, and the information database under construction promises to be a powerful tool in our understanding of cell surface molecules and their functions.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09250-28 EIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Cell-Mediated Cytotoxicity

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

PI: John Wunderlich Senior Investigator EIB, NCI

Others: David 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:

0.5

## 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.)

Last year's summary reported that cytolytic mouse T cells were successfully retargeted with bispecific antibodies, so that they lysed, in vitro, syngeneic breast cancer cells induced by the mammary tumor virus. The bispecific antibody reacted with a triggering site on the surface of T cells a CD3 component within the T-cell receptor complex, and with a virus-related antigen expressed on the surface of the tumor cells. Two requirements for the antitumor activity were that 1) the T cells had to be preactivated, and 2) the antibodies comprising the bispecific reagent had to be crosslinked and not simply mixed together. We preactivated the T cells by culturing splenocytes from normal donors with irradiated allogeneic cells, bacterial lipopolysaccharide, and recombinant IL-2.

Preactivated mouse spleen cells also blocked the growth of syngeneic breast cancer cells in tumor neutralization (Winn) assays in vivo, if retargeted against the tumor cells with the bispecific antibodies.

Because the project was closed out this year, we focused our effort on confirming selected observations described above.

## Project Description

Major Findings:

The focus of this laboratory has been on antitumor cytotoxic effector cells and factors that influence their generation. This was the final year of this project, and our we focused our effort on confirming selected observations associated with the establishment of a mouse model for retargeting cytotoxic T cells so that they react against breast cancer.

Retargeting T lymphocytes against tumor cells.

Two fundamental requirements of cytotoxic T cells are (1) that they express receptors capable of triggering the release of cytotoxic molecules following ligand binding and (2) that they be sufficiently activated to produce and, following triggering, release the effector molecules that mediate cytotoxicity. With bispecific antibodies we have succeeded in giving the T cell receptors the ability to recognize tumor cells, as described in previous progress reports. Recently we have focused on using bispecific antibodies to retarget T cells, so that they react against breast cancer in a mouse model.

Breast cancer, induced by the murine mammary tumor virus (MTV), appears spontaneously in more than 75% of females from some strains of mice, such as MTV-positive C3H. Also, a variety of mammary tumor cell lines are available, as are monoclonal antibodies that react with MTV components expressed on the cell-surface of mammary tumor cells.

Earlier, we found that mouse cytolytic T cells, irrespective of their native specificities, can be retargeted to react selectively with mouse breast cancer cells induced by the mouse mammary tumor virus. Retargeting was achieved with bispecific antibodies that recognized the T-cell receptor and the viral envelope glycoprotein, gp52. Gp52 is expressed on the surface of MTV-induced tumor cells.

We found using flow cytometry that gp52 was expressed on most (perhaps all) MTV-induced primary tumor cells but not on splenocytes, with the exception of about 5% of cells that were probably a subpopulation of B lymphocytes. To disperse the tumor cells, we used a combination of collagenase, hyaluronidase, and DNase that did not appear to affect the expression of cell-surface gp52. We also found that splenic cytolytic T cells, nonspecifically preactivated in vitro, lysed MTV-induced primary tumor cells when retargeted against gp52 with bispecific antibodies. The retargeted cytolytic T cells did not affect splenocytes from tumor-bearing mice or tumor cells that did not express gp52.

Retargeted mouse T cells were also effective in blocking the growth of breast cancer cells in vivo in tumor neutralization (Winn) assays. The antitumor activity required a combination of 1) preactivated T cells and 2) bispecific antibodies rather than the parent antibodies simply mixed

together. We preactivated T cells by culturing splenocytes with a mixture of alloantigens, bacterial lipopolysaccharide, and low doses of recombinant IL-2.

Work this year confirmed the ability of retargeted mouse T cells to block tumor growth in tumor neutralization assays.

Proposed Course

The project has ended.

Publications:

Wunderlich JR, Mezzanzanica D, Garrido MA, Neblock DS, Daddona PE, Andrew SM, Zurawski Jr., VR, Canevari S, Colnaghi MI, Segal DM.: Bispecific antibodies and retargeted cellular cytotoxicity: novel approaches to cancer therapy. *Int J Clin Lab Res* 1992;22:17-20.

Segal DM, Qian J-h, Mezzanzanica D, Garrido MA, Titus JA, Andrew SM, George AJT, Jost CR, Perez P, Wunderlich JR.: Targeting of anti-tumor responses with bispecific antibodies. *Immunobiology* 1992;185:390-402.

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Segal DM, Qian JH, Titus JA, Moreno MB, George AJ, Kurucz I, El-Gamil M, Wunderlich JR.: Targeted cytokine production. *Int J Cancer Suppl* 1992;7:36-38.

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

PROJECT NUMBER

Z01 CB 09251-22 EIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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, PI: P.A. Henkart Senior Investigator EIB, NCI

Others: H. Nakamura Visiting Fellow EIB, NCI  
 H. Park Biologist EIB, NCI  
 M. Williams IRTA Fellow EIB, NCI  
 R. Blumenthal Microbiologist EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

Lymphocyte Cytotoxicity Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4.2

PROFESSIONAL:

2.2

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- (a) Human       (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 test the granule exocytosis model for lymphocyte cytotoxicity, we have examined the cytotoxic activity of the rat mucosal mast cell tumor line RBL after transfection with genes for cytotoxic lymphocyte granule components. We have constructed triple, double, and single RBL transfectants expressing cytolysin (cy) and the granule serine proteases granzyme A (gza) and granzyme B (gzb). RBL-cy transfectants show only modest cytotoxicity on tumor targets, with no accompanying target DNA degradation. While RBL-gza transfectants express gza at levels comparable to cloned CTL and secrete it in response to IgE cross-linking, they have no cytotoxic activity detectable. RBL-cy-gza transfectant clones showing good expression of both these granule components showed cytolytic activity comparable to RBL-cy on RBC targets, but were greater than 3x more lytic on three different tumor targets. This cytotoxicity is accompanied by target DNA fragmentation. To confirm that killer cell granzymes need to enter the target cell, we loaded target cells with the macromolecular protease inhibitor aprotinin by osmotic lysis of pinosomes. Compared to BSA-loaded targets or unloaded targets, aprotinin-targets were less susceptible to lysis and DNA breakdown by CTL and RBL transfectants expressing granzyme A. However, RBL transfectants expressing only cytolysin lysed BSA-loaded and aprotinin-loaded targets with equal efficiency. As a direct test of the ability of proteases to induce cytotoxicity when introduced into the cytoplasm of a target cell, we have "injected" various proteases into tumor cells using osmotic lysis of pinosomes. The endoproteases trypsin, chymotrypsin, and proteinase K were all found to lyse several different types of tumor cells in a dose dependent manner, as measured by <sup>51</sup>Cr release. This death was generally apoptotic by morphological criteria and DNA fragmentation.

## Project Description

Major Findings:

In order to test the granule exocytosis model for lymphocyte cytotoxicity, we have examined the cytotoxic activity of the rat mucosal mast cell tumor line RBL after transfection with genes for cytotoxic lymphocyte granule components. In particular we have sought to test whether the granule serine proteases known as granzymes play a role in target cell damage. We have constructed triple, double, and single RBL transfectants expressing cytolysin (cy) and the granule serine proteases granzyme A (gza) and granzyme B (gzb). RBL-cy transfectants show only modest cytotoxicity on tumor targets, with no accompanying target DNA degradation. While RBL-gza transfectants express gza at levels comparable to cloned CTL and secrete it in response to IgE cross-linking, they have no cytotoxic activity detectable. RBL-cy-gza transfectant clones showing good expression of both these granule components showed cytolytic activity comparable to RBL-cy on RBC targets, but were greater than 3x more lytic on three different tumor targets. This cytotoxicity is accompanied by target DNA fragmentation. To confirm that killer cell granzymes need to enter the target cell, we loaded target cells with the macromolecular protease inhibitor aprotinin by osmotic lysis of pinosomes. Compared to BSA-loaded targets or unloaded targets, aprotinin-targets were less susceptible to lysis and DNA breakdown by CTL and RBL transfectants expressing granzyme A. However, RBL transfectants expressing only cytolysin lysed BSA-loaded and aprotinin-loaded targets with equal efficiency. As a direct test of the ability of proteases to induce cytotoxicity when introduced into the cytoplasm of a target cell, we have "injected" various proteases into tumor cells using osmotic lysis of pinosomes. The endoproteases trypsin, chymotrypsin, and proteinase K were all found to lyse several different types of tumor cells in a dose dependent manner, as measured by <sup>51</sup>Cr release. This death was accompanied by DNA fragmentation to a variable extent, depending on the target cells. This protease-induced nuclear damage has the morphological characteristics of apoptosis. Cytotoxicity by intracellular proteolysis is not inhibited by agents which block programmed cell death.

Proposed course:

We are currently analyzing RBL clones transfected with granzyme B cDNA as well as those granule components previously studied. We will examine whether the triple transfectants have cytolytic activity on tumor targets comparable to CTL.

Publications:

Shiver JW, L Su, Henkart PA. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. Cell 1992;71:315-322.



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

PROJECT NUMBER

Z01 CB 09254-19 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

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

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,  
 PI: D. M. Segal Section Chief EIB, NCI  
 Others: C. Sconocchia Visiting Fellow EIB, NCI  
 M. Mareno Special Volunteer EIB, NCI  
 J. R. Wunderlich Senior Investigator EIB, NCI

COOPERATING UNITS (if any)

J. Yun Tso, Staff Scientist, Protein Design Labs, Inc., Mountain View, CA.

LAB/BRANCH  
 Experimental Immunology Branch

SECTION  
 Immune Targeting Section

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
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CHECK APPROPRIATE BOX(ES)

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

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

A murine breast cancer model has been developed for measuring the ability of targeted effector cells to eradicate primary and transplanted mammary tumors. Bispecific antibodies containing anti-CD3 crosslinked to antibodies against gp52 from the mouse mammary tumor virus bind specifically to spontaneous or cultured mammary tumor cells, and induce murine T cells to lyse such cells in vitro and block the growth of subcutaneous tumor transplants in vivo. A genetically engineered bispecific F(ab')<sub>2</sub> construct has been produced that has the same in vitro targeting activity as conventionally prepared bispecific antibodies.

By using anti-CD44 containing bispecific antibodies, we have found that CD44 is a cytotoxic triggering molecule on a subset of human PBL. Cells mediating such targeted lysis reside in the LGL population, are CD56+, and are activated by IL-2.

## Project Description

Major FindingsBreast cancer model.

Bispecific antibodies that link target cells to triggering structures on cytotoxic cells induce these cells to lyse the bound target cells. Such "targeted cytolysis" has been achieved in vitro using T cells, NK cells, monocytes, macrophages, and granulocytes as effectors, and many different types of targets, including tumor cells and virally infected cells. Targeted human T cells block the growth of human tumor cells in nude mice and syngeneic B cell lymphomas in normal mice. Recently, we have developed a totally syngeneic murine breast cancer model to test the ability of bispecific antibodies to eradicate solid tumors in vivo. Mouse mammary tumors are virally induced and tumor cells express viral coat proteins on their surfaces. Monoclonal antibodies against the gp52 viral coat protein bind specifically to mouse mammary tumor cells, and we have crosslinked one anti-gp52 mAb, P2AE12, to 2C11 (anti-murine CD3) by conventional chemical methods. Activated murine T cells targeted with 2C11 x P2AE12 lyse primary mammary tumor cells and cultured cell lines and block the subcutaneous growth of tumor in syngeneic mice in a Winn-type assay. In order to carry out in vivo studies, we have, in collaboration with J. Tso at Protein Design Labs, developed a genetically engineered bispecific antibody, as a prototype of one that could eventually be used to treat cancer patients. This bispecific antibody consists of 2C11 Fab linked to P2AE12 Fab. The Fab proteins are produced in mammalian cells using expression vectors containing the appropriate Fab construct. Each Fab is produced independently, with one Fab containing a fos leucine zipper at its C terminus, and the other a jun zipper. In addition, each Fab contains a portion of hinge region with 3 sulfhydryl residues. When the two Fab proteins are mixed, they preferentially form the bispecific F(ab')<sub>2</sub>. So far, approximately 80 µg of material has been produced, and has been shown to be active in a lytic assay using activated mouse spleen cells as effectors, and a mammary tumor line as targets.

Targeting with CD44

It has recently been reported that mouse T cell clones can be induced to lyse FcR<sup>+</sup> target cells with an anti-CD44 monoclonal antibody. This suggests that CD44, an adhesion molecule that binds to hyaluronic acid and is thought to be involved in lymphocyte homing, can trigger lysis when it binds to cells expressing its ligand. In order to see whether this would apply to normal human lymphocytes, we made a bispecific antibody that binds to both human CD44 and a target cell antigen. This antibody induced IL-2 activated human PBL to specifically lyse target cells. The targetable activity resides in a low buoyant density fraction that is CD56<sup>+</sup>. This subset contains both CD16<sup>+</sup> NK cells, and CD3<sup>+</sup> T cells. We are currently trying to determine which of these subsets has lytic activity targetable through CD44.

Proposed course of projectMouse mammary tumor model

We plan to use the mouse mammary tumor model to study the ability of bispecific antibodies to home to and eradicate primary and secondary solid tumors in normal mice. For these studies we will use the genetically engineered bispecific F(ab')<sub>2</sub>. We should have sufficient material for these studies in the next few weeks. We will use mice bearing primary tumors, subcutaneous transplants, and lung metastases following iv injection of tumor, as test systems. 64PT, a BALB/c MTV<sup>+</sup> tumor line, grows well subcutaneously in BALB/c mice and will therefore be one of the tumors we will use in our studies. We will first see how the bispecific antibodies home in tumor bearing mice, and whether they will have an effect upon T cell homing. This will be done using <sup>125</sup>I-labeled bispecific antibody, and <sup>111</sup>In-labeled splenocytes. We will test the effects of ip and iv administration of antibody, and will sacrifice mice and measure organ distribution of isotope following administration. Next, we will test bispecific antibodies for the ability to abrogate tumor growth. This will be done by following subcutaneous growth of established tumor transplants, numbers of lung metastases following iv injection of tumor, survival times of mice bearing tumor transplants, and tumor size and survival of mice with primary mammary tumors. Treatment will involve iv and ip injection of bispecific antibodies at various stages of tumor growth, or treatment of mice with ex-vivo activated cells and bispecific antibodies. Finally, as these studies proceed, we will try to gain some information on the mechanism by which targeted T cells block tumor growth. This will be done by looking for the blockage of bystander tumor growth, and by injecting antibodies against cytokines such as TNF- $\alpha$  and IFN- into tumor bearing mice following treatment with the bispecific antibody.

CD44 targeting

Our immediate plans are to determine which cells in PBL are targetable through CD44. To do this we will use the FACS to sort various subsets of cells and test them for CD44 targetable activity. We also want to examine in detail what the activation requirements of these cells are, and see if we can produce human T cell lines or clones that can mediate this activity. Following those studies, we plan to see whether a particular isoform of CD44 is used in the lytic process, and how this molecule becomes a cytotoxic trigger in certain cell populations.

Publications:

Segal DM, Qian J-h, Mezzanzanica D, Garrido MA, Titus JA, Andrew SM, George AJT, Jost CR, Perez P, Wunderlich JR. Targeting of anti-tumor responses with bispecific antibodies. *Immunobiol* 1992;185:390-402.

Segal DM, Urch CE, George AJT, Jost CR. Bispecific antibodies in cancer treatment: in *Biologic Therapy of Cancer Updates*, vol 2. VT DeVita, S Hellman, SA Rosenberg, eds JB Lippincott Co., Philadelphia. 1992;pp 1-12.

Segal DM, Qian J-h., Titus JA, Moreno MB, George AJT, Jost CR, Kurucz I, El-Gamil M, Wunderlich JR. Targeted cytokine production. *Int J Cancer* 1992(suppl 1):36-38.

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

PROJECT NUMBER

Z01 CB 09255-19 EIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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, PI:

S. O. Sharrow Senior Investigator EIB, NCI

Others: L. G. Granger Biologist EIB, NCI  
 C. L. Johnson General Fellow EIB, NCI  
 Members of the Experimental Immunology Branch, NCI (see text)

COOPERATING UNITS (if any)

A. Schultz, L. Barden, R. Tate, and J. Powell, CSL, DCRT; M.C. Udey, DB, DCBDC, NCI, C.C. Ting, OD, DCBDC, NCI.

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immune Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.75

PROFESSIONAL:

1.0

OTHER:

1.75

CHECK APPROPRIATE BOX(ES)

- (a) Human  (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 multiple research projects for more than 40 investigators. These investigations involve quantitative single cell analysis of parameters associated with cells freshly prepared from different species/tissues, as well as a spectrum of in vitro cultured cells. Basic research support is provided to members of the EIB as well as to other investigators within DCBDC. Currently supported projects include, but are not limited to, the following areas of study: a) in vivo and in vitro analyses of intra-cellular signalling via T cell surface molecules, b) analyses of cellular defects in animals with genetic or induced immune dysfunction; c) studies of the pathogenesis of graft-versus-host disease; d) analyses of the coordinate cell surface expression of cell adhesion molecules; e) investigations of T cell ontogeny and differentiation; f) studies of mechanisms of T cell repertoire generation; g) analyses of expression of transplantation antigens; h) investigations of mechanisms involved in 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 two user-operated single beam flow cytometers, maintains a reagent bank which supplies reagents to users of the flow cytometers, and provides consultation in flow cytometry techniques, protocol design, reagent selection, and data analysis. This report summarizes findings only in selected project areas which utilized the dual-beam flow cytometer, and emphasizes those aspects most heavily supported by the use of flow cytometry analysis.

Dr. A. Singer and colleagues have continued to utilize flow cytometry analysis in studies of the relationships between T cell receptor expression and intrathymic T cell differentiation. In these studies, they have characterized thymocytes from C.B-17/scid mice which are unable to productively rearrange antigen receptor genes, and thus generally fail to express T cell receptors. These efforts depend upon multi-color analysis and the ability to analyze low frequency events. It was found that SCID (severe-combined immunodeficiency disease) thymocytes are predominantly CD5<sup>-</sup>dull, Thyl.2<sup>+</sup>, CD4<sup>-</sup>CD8<sup>-</sup> and contain both IL-2r<sup>+</sup> and IL-2r<sup>-</sup> cells. This phenotype mimics that of a population of normal thymocytes known to contain precursors of mature thymocytes. It was also found that the presence of T cell receptor bearing thymocytes in SCID thymii was associated with further differentiation and expression of CD4 and/or CD8 accessory molecules by SCID thymocytes which themselves did not express T cell receptor. These results suggest that during thymic development, the expression of molecules critically important to T cell differentiation of immature cells is controlled by other T cells which themselves express T cell receptor. Further studies have demonstrated that CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from TCRV $\beta$ 8-transgenic SCID mice express low, but detectable levels of cell surface CD3 components which are associated with disulfide-linked TCR $\beta$  dimers. In contrast, mature CD4 or CD8 single positive T cells in these same thymii expressed high surface levels of CD3 components associated with functionally competent disulfide-linked heterodimers consisting of transgenic TCR $\beta$  chains and endogenous TCR $\alpha$  chains. Structurally distinct TCR complexes are thus expressed by TCRV $\beta$ -transgenic SCID thymocytes depending upon their differentiation status. These studies confirm an obligatory role during thymus development for surface expression of fully assembled TCR complexes.

Dr. A. Singer and colleagues have also utilized flow cytometry analysis in studies of minor lymphocyte stimulatory (Mls) expression in SCID mice, as well as in another mouse model of immune deficiency, X-linked immunodeficiency (xid). Expression of Mls determinants is not well understood, although it is known that these antigenic determinants are derived from mammary tumor proviral transcripts and are expressed by B lymphocytes. While SCID mice are devoid of all B and T lymphocytes, xid animals lack only a subset of mature B cells. Flow cytometric analysis was used to characterize the Mls-dependent T cell repertoire in these animals. It was found that Mls<sup>a</sup>-reactive V $\beta$ 6<sup>hi</sup> T cells were deleted in the thymi of male xid animals and that Mls<sup>c</sup>-reactive V $\beta$ 3<sup>-hi</sup> AKR/J thymocytes and spleen cells were deleted in AKR/J --> SCID chimeras. These data, in conjunction with functional analyses, demonstrate that functional Mls expression occurs in the absence of mature B and T cells.

Flow cytometry is also used by Dr. A Singer and colleagues in studies of mechanisms of murine intra-thymic T cell differentiation. For example, these investigators have characterized  $CD4^+CD8^+$  transitional thymocytes which give rise to  $CD4^+CD8^+$  thymocytes. It was found that these  $CD8^+$  transitional cells expressed low levels of functional T cell receptor. Most importantly, the in vitro transition of  $CD8^+TCR^{lo}$  cells into  $CD4^+CD8^+$  double positive thymocytes was inhibited by crosslinking with anti-TCR antibodies. The abrogation of increased cell surface expression of CD4 was due to a failure to increase CD4 protein synthesis. These results suggest that TCR-mediated signals may play a role in T cell differentiation prior to the  $CD4^+CD8^+$  double positive differentiation stage and raise the possibility that negative selection of the T cell repertoire could occur at an earlier stage of development than was previously thought. These studies have further shown that cross-linking of T cell receptor molecules on double positive thymocyte precursors eliminated messenger RNAs not only for the co-receptor molecules, CD4 and CD8, but also for the recombination activating genes 1 and 2. This post-transcriptional regulation, induced via TCR-mediated signals, required protein synthesis and was developmentally regulated. These findings not only have identified a novel mechanism for regulation via TCR-signalling, but also have contributed to our understanding of early thymocyte development.

Dr. Richard Hodes and colleagues have utilized quantitative multi-color immunofluorescence and flow cytometry to characterize expression of different CD45 isoforms by subpopulations of activated murine B cells. Surface expression of variable exon specific CD45 determinants was analyzed on resting B cells and on B cells activated by LPS or by IL-5. These analyses revealed that cell surface expression of different forms of the CD45 molecule is altered on activated B cell subpopulations and that the pattern of CD45 isoform expression is specific to the stimuli utilized. Combined with biochemical and PCR RNA analyses, these studies demonstrate that CD45 isoform analyses provide a novel tool for elucidation of the mechanisms and consequences of B cell activation.

In other studies, Dr. Hodes and colleagues have continued to utilize flow cytometry for an extensive series of studies of expression of the repertoire of T cell receptor genes in inbred mouse strains, recombinant inbred strains, backcross genetic studies, radiation bone marrow chimeras, murine retroviral infection models and endogenous murine mouse mammary tumor models. These murine T cell receptor repertoire investigations were critically dependent upon the ability of flow cytometry to provide reliable, precise measurements of low frequency subpopulations. The studies have focussed on: a) the mechanisms by which superantigens play a role in T cell receptor repertoire selection; b) characterization of associations between Mls stimulatory antigen expression and the expression of specific T cell receptor  $V\beta$  gene products; c) analysis of negative selection of the T cell repertoire, d) analysis of ligands, including mammary tumor virus antigens and xenoantigens, which mediate  $V\beta$ -specific negative and positive selection of the T cell repertoire; and e) analysis of the repertoire of T cells responsive to infectious retrovirus and syngeneic tumors.

Dr. Hodes and colleagues have found that there is an association between  $Mls^c$  expression and deletion of  $V\beta 3$  positive,  $Mls^c$  reactive T cells in both the thymus and periphery. It was further found that  $Mls^c$  expression is under multi-gene control, emphasizing the importance of non-major histocompatibility (MHC) antigens in selection of the T cell receptor repertoire. Negative selection of

the T cell repertoire via clonal deletion was found to be thymus dependent and did not occur in athymic nude mice. Analyses of the self-ligands responsible for deletions found that both MHC and non-MHC antigens appear to be involved. Based upon these analyses, new superantigens have been proposed on the basis of clonal deletion of  $V\beta 11$ ,  $V\beta 12$ , and  $V\beta 5$  and that these ligands behave as a novel minor lymphocyte-stimulating (Mls) determinant(s), Mls<sup>F</sup>. Genetic analyses demonstrate that at least three known mouse mammary tumor pro-viruses (MMTV), Mtv-8, Mtv-9 and Mtv-11 are involved in the expression of Mls<sup>F</sup> gene products. To further understand the requirement for superantigen properties in MMTV, a highly tumorigenic strain of MMTV was analyzed for its effect of TCR  $V\beta$  expression by T cells. It was found that exposure of newborn mice to milk-born virus resulted in marked, MHC-dependent, deletion of  $V\beta 2^+$   $CD4^+$  T cells, while in adult animals exposure to virus-containing milk induced specific expansion of these same T cells. These findings support a critical role for superantigen-mediated T cell activation in MMTV infection and tumorigenesis. These studies by Dr. Hodes and colleagues have recently been extended to include analyses of TCR  $V\alpha/V\beta$  pairing. It has been found that  $V\alpha/V\beta$  pairing is non random and that there exist strain specific differences in specific  $V\alpha/V\beta$  pairs. These results suggest that detailed analysis of  $V\alpha/V\beta$  TCR pairs will provide a novel approach for investigation of T cell repertoire selection by self antigens.

Dr. Ronald Gress and colleagues utilized flow cytometry in a series of studies of mechanisms of bone marrow transplantation in mice, monkeys and humans. Flow cytometric analyses are used to characterize immune cell reconstitution, to analyze cellular components which contribute to rejection versus engraftment of stem cell populations used in reconstitution, and to evaluate immunosuppressive therapies used to prevent graft rejection. In a study of autologous transplantation in rhesus monkeys, it was found that the pattern and time course of reconstitution of T lymphocytes was dependent upon the number of residual T cells in the infused bone marrow, rather than upon the marrow dose or efficacy of general hematopoietic reconstitution. Dr. Gress' laboratory has also investigated the regenerative and progenitor potential of mature murine T cell populations utilizing an irradiation bone marrow chimera model in which allotypic markers are used to distinguish T cells arising from stem cells from those which are derived from mature T cell populations. The observation that mature T cells contribute to immune reconstitution after bone marrow transplantation was confirmed in this study, which also demonstrated that expansion of mature T cells occurs during reconstitution. These studies have further demonstrated that T cell reconstitution after transplantation with T cell-containing marrow involves three T-cell precursor sources: 1) the marrow stem cells themselves; 2) the infused T cells; and 3) residual T cells in the irradiated host. It was found that each pool gives rise to distinct progeny, and that under conditions of limited thymic function, the infused T cells dominate T cell reconstitution. These studies are important to our understanding of the mechanisms of bone marrow engraftment and rejection, especially as applied to clinical problems.

Dr. Stephen Shaw and colleagues have received flow cytometry support for an extensive investigation which involves characterization of cell surface molecules which are differentially regulated on human T cells. These studies utilize quantitative multi-color immunofluorescence analyses of surface antigens on T cell subsets from a variety of organs. The analyses involve quantitative

assessments of coordinate versus non-coordinate expression of a wide variety of molecules with varying biological functions, including T cell receptor accessory molecules, CD45 isoforms, VLA integrins, and a variety of other cell adhesion ligands and receptors. Initial investigations demonstrated that functionally distinct T cell subsets express quantitatively different surface levels of multiple biologically functional molecules. Studies which focussed on the CD4<sup>+</sup> T cell subset have demonstrated that unexpectedly large numbers of cell surface antigens are coordinately up-regulated or down-regulated as T cells differentiate between "naive" (not previously stimulated) and "memory" (previously activated) states of maturation.

One of these investigations by Dr. Shaw has characterized, on T cells, the expression and function of the VLA ( $\beta$ 1) integrin receptor family which is thought to be important in T cell migration. It was found that memory T cells express 3-4 fold more cell surface VLA-4, VLA-5, and VLA-6 than do virgin T cells, a finding that correlated with separate (non-flow cytometry) binding studies which demonstrated increased binding of memory cells to the extracellular matrix proteins laminin and fibronectin. Systematic analyses of expression of 5 VLA integrin chains with simultaneous analyses of CD45RA and CD45RO isoforms have demonstrated that regulation of VLA- $\alpha$ 4 can occur independently of VLA- $\alpha$ 3, VLA- $\alpha$ 5, VLA- $\alpha$ 6 and VLA- $\beta$ 1. It was also found that at least three subsets of memory cells can be discriminated on the basis of VLA- $\alpha$ 4 expression ( $\alpha$ 4-negative,  $\alpha$ 4 $\beta$ 7-high, and  $\alpha$ 4 $\beta$ 1-high). These analyses are being extended to T cells derived from sites other than peripheral blood, including spleen, lymph node, tonsil and gut lamina propria lymphocytes. To date, more than 400 different monoclonal antibodies have been utilized in these multi-variable analyses. In addition, the flow cytometry lab has provided support to Dr. Shaw in his efforts on behalf of the 5th International Workshop on Leukocyte Differentiation Antigens. Utilizing specialized software developed by the flow cytometry lab, the EIB VAX/VMS computer systems have served as the INTERNET connection for laboratories participating in the workshop, allowing direct electronic data transfer to the EIB of flow cytometry raw data files.

Dr. Mark Udey (Dermatology Branch, DCBDC, NCI) and colleagues have utilized flow cytometry to investigate the expression of cadherin molecules, known to be involved in keratinocyte-keratinocyte interactions, on epidermal Langerhans cells which are hematopoietic, antigen presenting cells of the skin. Preparative flow cytometry was used to purify epidermal Langerhans cells and keratinocytes in order to characterize mRNA of these cells types using PCR analysis. It was found that while Langerhans cell-derived mRNA was markedly enriched in I-A  $\alpha$  chain compared to mRNA from keratinocytes, mRNA from both cell types contained comparable amounts of E-cadherin PCR products. These data showed that cell surface E-cadherin detected on epidermal Langerhans cells by immunofluorescence was indeed synthesized by these cells. Because this is the first demonstration of expression of cadherins by hematopoietic cells, this study raises the novel possibility that cadherins may play a role in leukocyte migration.

S. Sharrow, L. Barden (CSL, DCRT) and colleagues have developed a user-friendly software system to provide automated cluster analysis and associated graphics techniques. The Cluster Analysis Program (CAP) provides methodology for analysis of multi-color immunofluorescence list mode flow cytometry data and



operates in the VAX/VMS environment. BETA-testing of CAP has been completed and the software is currently available for use by EIB investigators and for release to other laboratories. S. Sharrow, J. Powell (CSL, DCRT) and colleagues have developed a flow cytometry version of the Laboratory Analysis Package (LAP) (originally developed by CSL, DCRT) for histogram analysis of flow cytometry data. BETA-test of Flow Cytometry LAP, which has been ported to the VAX/VMS environment, has been completed and this package is also available for release to other laboratories.

#### Publications:

Horgan KJ, Tanaka Y, Shaw S. Post-thymic differentiation of CD4 T lymphocytes: Naive vs memory subsets and further specialization among memory cells. *Chem Immunol* 1992;54:72-102.

Roberts JL, Abe R, Shores EW, Singer A. Expression of Mls determinants in mice exhibiting the severe combined immunodeficiency (scid) mutation or X-linked immunodeficiency (xid) defect. *J Immunol* 1992;149:1577-1582.

Ting CC, Hargrove ME, Liang SM, Liang CM, Sharrow SO. Dichotomy of glutathione regulation of the activation of resting and preactivated lymphocytes. *Cell Immunol* 1992;142:40-53.

Hathcock KS, Hirano H, Murakami SA, Hodes RJ. CD45 expression by B cells. Expression of different CD45 isoforms by subpopulations of activated B cells. *J Immunol* 1992;149:2286-2294.

Takahama Y, Shores EW, Singer A. Negative selection of precursor thymocytes before their differentiation into CD4<sup>+</sup>CD8<sup>+</sup> cells. *Science* 1992;258:653-656.

Takahama Y, Singer A. Post-transcriptional regulation of early T cell development by T cell receptor signals. *Science* 258:1456-1462.

Horgan KJ, Ginther Luce GE, Tanaka Y, Schweighoffer T, Shimizu Y, Sharrow SO, Shaw S. Differential expression of VLA- $\alpha$ 4 and VLA- $\beta$ 1 discriminates multiple subsets of CD4<sup>+</sup>CD45RO<sup>+</sup> "Memory" T cells. *J Immunol* 1992;149:4082-4087.

Abe R, Foo-Phillips M, Granger LG, Kanagawa O. Characterization of the Mls<sup>f</sup> system. I. A novel polymorphism of endogenous superantigens. *J Immunol* 1992;149:3429-3439.

Foo-Phillips M, Kozak CA, Principato, Abe R. Characterization of the Mls<sup>f</sup> system. II. Identification of mouse mammary tumor virus proviruses involved in the clonal deletion of self-Mls<sup>f</sup>-reactive T cells. *J Immunol* 1992;149:3440-3447.

Tang A, Amagai M, Granger LG, Stanley JR, Udey MC. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature* 1993;361:82-85.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09257-18 EIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## 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:	Stephen Shaw	Section Chief	EIB, NCI
Others:	Gale Ginther Luce	Chemist	EIB, NCI
	John Hallam	Biologist	EIB, NCI
	Tamas Schweighoffer	Visiting Fellow	EIB, NCI
	Marina Giunta	Visiting Fellow	EIB, NCI
	David Adams	Visiting Associate	EIB, NCI
	Eva Tolosa	Special Volunteer	EIB, NCI
	MaryAnne Liotta	IRTA	EIB, NCI

## COOPERATING UNITS (if any)

US Army Medical Research Institute on Infectious Diseases: Art Anderson  
 Department of Pathology, University of Birmingham, UK: Stefan Hubscher  
 The Laboratory of Immunoregulation, NIAID, NIH: Ulrich Siebenlist

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

Human Immunology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

8.0

## PROFESSIONAL:

8.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

B

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

Our studies continue to emphasize characterizing cell surface molecules which facilitate T cell function, as well as defining their regulation with T cell differentiation. Major advances have been made this year in the critical area of T cell adhesion to endothelium. Our work strengthens the model that binding occurs via a cascade of at least 3 steps: tether, trigger, strong adhesion. The triggering step for T cells has been the least well defined. We have discovered two "pro-adhesive" cytokines which promise to be physiologic triggers: MIP-1 $\beta$  and HGF. MIP-1 $\beta$ : 1) induces T cell adhesion to the endothelial ligand VCAM-1; 2) can be retained on proteoglycan to mediate its adhesion-induction; 3) is found at the endothelial surface in tonsil and lymph node; and 4) is chemotactic for T cells. Although, hepatocyte growth factor (HGF) is structurally completely different from MIP-1 $\beta$  and previously had no immunologic relevance, we find it is functionally similar: strongly chemotactic for T cells as well as inducing T cell adhesion. Immunohistologic studies identify HGF on endothelium at sites such as inflamed liver. We propose that these factors are retained at the endothelial surface by binding to proteoglycan. We have developed a broader physiologic model that such pro-adhesive cytokines are delivered to endothelium by a specialized fibroblastic reticular conduit system. In studies of the integrins which mediate strong adhesion to endothelium. Flow cytometry and 2D-gel analysis identifies predominantly  $\alpha 6\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 4\beta 7$ , and  $\alpha 5\beta 1$  on T cells. Low uniform expression of integrins is seen on naive cells and higher more selective expression on memory cells, including a newly defined subset of CD4 cells virtually negative for  $\alpha 4$ . Finally, we have organized a major component of the upcoming Fifth International Workshop on Leukocyte Differentiation Antigen which will determine the level of expression of virtually all known leukocyte surface molecules on cells of many lineages.

## Project Description

Major Findings:

Since T cell surveillance depends on movement from blood into tissue and back again, rapid, efficient and selective T cell adhesion to vascular endothelium is essential. This adhesion involves a multistep cascade clarified by a recent consensus model, emerging from results in our lab and a number of other labs studying lymphocytes, granulocytes and platelets: 1) initial tethering by selectin-mediated interactions; 2) triggering of adhesive function of T cell integrins by ligands at or near the endothelial surface; and 3) strong adhesion mediated by T cell integrins.

We have previously made contributions to understanding the strong adhesion step. However, we have recently turned more of our attention to the triggering step, which in our view is the major unknown in this process. Adhesion cannot occur without it, but no physiologic mediators had been defined. We had thought primarily of cell surface molecules, including particularly CD31 based on our previous studies. Although others' data regarding IL8-induction of granulocyte adhesion suggested cytokines as candidates for physiologic adhesion-induction, we disliked that idea on theoretical grounds -- cytokines would be washed away, and therefore not be likely to reach effective local concentrations. However, in the last year or two we have changed our mind to view cytokines as pivotal elements which make sense because we now believe that evolution has engineered a "trick" to deal with washout. We propose that the trick is immobilization of cytokines on the endothelial surface. Proteoglycans are present on the luminal surface of endothelium and are known to facilitate localization of other proteins such as protease inhibitors at the vessel wall.

To explore potential involvement of cytokines in regulation of T cell adhesion we have screened more than a dozen cytokines for their involvement both in inducing integrin-mediated adhesion and in inducing T-cell chemotaxis. We view both as relevant to T cell recruitment, and think it is likely that overlapping biochemical pathways are involved in adhesion-induction and chemotaxis. Among the cytokines tested, we found that the majority have chemotactic effects on T-cells. This finding indicates that T cells are responsive to a very rather large number of soluble cues in their microenvironment.

Among the cytokines surveyed, only two had the property in which we were most interested: the ability to induce T cell adhesion. MIP-1 $\beta$  showed the most pronounced induction of adhesion; HGF did also, albeit less strongly. MIP-1 $\beta$  is a member of the "chemokine" family which includes multiple chemotactic cytokines such as IL-8, MCP and RANTES. MIP-1 $\beta$  induced augmented adhesion of CD8 T cells to VCAM-1 in all donors tested and to the extracellular matrix ligand fibronectin (FN) in most donors. MIP-1 $\beta$  augmentation of adhesion occurred predominantly on CD8 rather than CD4 cells. The induction of adhesion of particular T cell subsets by specific cytokines would contribute flexibility and selectivity to the process of lymphocyte recruitment.

Chemokines like MIP-1 $\beta$  possess GAG-binding sites and bind heparin. There are numerous biological systems in which cytokines interact in functionally important ways with proteoglycans in extracellular matrix and on cell surfaces. We propose a novel variation, namely that proteoglycans on an endothelial cell can bind and "present" cytokines to passing leukocytes. We created a biochemically defined model system to explore the hypothesis : 1) Proteoglycan (either heparin-BSA as a model proteoglycan or CD44 as a purified cell surface proteoglycan) and VCAM-1 (an endothelial ligand for VLA-4) were co-immobilized on plastic; 2) MIP-1 $\beta$  was added and then washed out to assess only the effect of immobilized cytokine; and 3) We assessed binding of resting CD8 T cells to all eight combinations of the three components: integrin ligand, proteoglycan and cytokine. Marked MIP-1 $\beta$ -specific augmentation of binding was observed only when both the proteoglycan and the integrin ligand were co-immobilized. Thus, MIP-1 $\beta$  can be retained by immobilized proteoglycan and induce T cell adhesion to integrin ligands on that same surface.

To explore whether cell surface proteoglycan could also "present" MIP-1 $\beta$  to T cells, we substituted CD44 for heparin-BSA conjugate in our model. The results with CD44, a cell surface proteoglycan, paralleled those with the synthetic proteoglycan. MIP-1 $\beta$ -specific augmentation of binding was consistently observed only when CD44 and the integrin ligand VCAM-1 were co-immobilized.

MIP-1 $\beta$  is produced in large amounts by monocytes, fibroblasts and lymphocytes early after activation in vitro; in vivo, it might bind to proteoglycan on the endothelial surface at inflammatory sites and be presented as an adhesion-inducing stimulus to passing leukocytes. Our hypothesis is supported by our detection of MIP-1 $\beta$  on the luminal surface of endothelium in lymphoid tissue as well as on endothelium at sites of inflammation such as tonsil.

The other cytokine which we found has the capacity to mediate both adhesion-induction and chemotaxis is HGF. HGF is a heparin-binding growth factor with structural homology to plasminogen. Among its pleiotropic effects, HGF effects on motility (both chemotaxis and scattering) are particularly pronounced, as reflected in its alternative name "scatter factor". Our first evidence that HGF is relevant to T cell recruitment was its ability to induce migration of resting normal human T cells. It induces typically a fourfold increase in T cell migration.

Within the T cell lineage there are many kinds of specialization of subsets. One which is particularly important in T cell migration is between naive cells and their more mature counterpart memory cells which have been previously activated by specific antigen. These subsets, operationally distinguished by isoforms of CD45, are markedly different in their cytokine profiles, activation requirements, adhesion capacity and in vivo migration. In our studies, HGF usually induces preferential migration of memory (CD45RA-) T cells. Because of the importance of subset-specific recruitment, we extended the studies of migration to include: the additional distinction of CD4 vs CD8 and another important pro-adhesive cytokine for T cells, MIP-1 $\beta$ . MIP-1 $\beta$  preferentially induces migration of naive cells. In contrast, HGF preferential induces migration of memory cells.

Since adhesion induction is the defining property of a pro-adhesive cytokine, we assayed HGF for its ability to induce adhesion of resting T cells to integrin ligands. We find that HGF induces modest [2-3 fold] induction of adhesion. As seen with chemotaxis, the response is restricted to memory cells. CD4 cells respond in preference to CD8 cells, which is consistent the relative paucity of memory cells in the CD8 population. This contrasts with the preferential adhesion of CD8 cells induced by MIP-1 $\beta$ .

Because the effects that we have seen with HGF and MIP-1 $\beta$  in traditional adhesion assays are modest in magnitude, we have sought additional assays in which to explore the effects of such cytokines on other aspects of T cell physiology known to be relevant to adhesion and motility. Since, actin polymerization is a dynamic process critical to both these functions, we analyzed F-actin distribution following T cell exposure to these cytokines. In contrast to most cells, resting T cells placed on fibronectin remain round and do not undergo changes in actin. However, rapidly after addition of MIP-1 $\beta$  or HGF T cells complex changes in F-actin occur, which are consistent are similar to those reported with motile cells such as neutrophils and amoebae after exposure to chemotactic factors. The rapid kinetics of F-actin changes can be quantitated by flow cytometric analysis. Marked increases in F-actin occur by 10-30 seconds in response to HGF. We interpret these actin changes as indicators of a physiologic pro-adhesive effect of these cytokines. They dramatically confirm the action of these cytokines on T cells and their rapidity approaches the time frame in which adhesion triggering must occur physiologically.

Our paradigm of adhesion induction by cytokine requires that the cytokine be retained at the endothelial surface, in many cases by proteoglycan. As incidental finding in previous studies, HGF can be detected on endothelium where it is expressed and also, probably, loosely bound by proteoglycan since it can be eluted from the vascular tree by hypertonic saline. A physiological role for HGF in recruiting T cells to site of inflammation is supported by our immunohistochemical studies demonstrating HGF expression on the luminal aspect of endothelium in inflamed liver. HGF is known to bind heparan and the low-affinity HGF receptor is believed to be located on heparan sulfate proteoglycans, which are abundant on the endothelial glycocalyx.

Thus structurally diverse cytokines, including HGF and MIP-1 $\beta$ , which are secreted by inflammatory cells beneath the endothelium might diffuse to the endothelial glycocalyx, bind to heparan sulfate proteoglycans and thereby present an immobilized ligand to passing lymphocytes. Recognition of immobilized cytokine by tethered lymphocytes could then activate T cell integrins within seconds inducing strong adhesion and subsequent migration into tissue where cytokine immobilized on proteoglycan could direct migration through the ECM. Such a model provides a powerful method for localizing and directing infiltrating lymphocytes. It is likely that this model applies to other cytokines/growth factors which bind heparin. Thus leukocyte migration might be controlled by many different cytokines immobilized and presented on proteoglycan each regulating the adhesion of particular leukocyte subsets and thereby providing specificity to the regulation of T cell-endothelial interactions.

Based on our findings with MIP-1 $\beta$  and HGF, we propose that cytokines present on the endothelial surface provide contribute to T cell recruitment. Although our present evidence is restricted to those two cytokines, we expect that a variety of others will also be involved. How do those cytokines reach the endothelial surface? Together with Art Anderson, we have formulated a broader concept of cytokine transport to the endothelial surface based on his prior studies, and our emerging data. We single out three remarkable anatomic features in LN which we propose maximize the role in lymphocyte recruitment of cytokines from afferent lymph: a transport system to bring soluble factors to the base of the HEV, specialized junctions between HEV to allow factors to reach the lumen, and a rich glycocalyx potentially able to bind and/or trap factors. We refer to this transport system as the "fibroblastic reticular cells (FRC) conduit system", which we believe is a specialized transport system within the LN for rapid and efficient delivery of cytokines and other soluble molecules directly to the HEV. Analogous structures are also present in other tissues.

Our studies of peripheral T cell differentiation have continued to emphasize expression of adhesion molecules, particularly integrins. Continued exploration with multicolor flow cytometry has confirmed and extended our concept that: 1) Naive cells are relatively homogenous phenotypically and express a relatively low uniform level of integrins, principally  $\alpha 4\beta 1$ ,  $\alpha 4\beta 7$ ,  $\alpha 6\beta 1$  and  $\alpha 5\beta 1$ . 2) Memory cells are very heterogeneous in phenotype and memory cell subsets have specialized adhesion phenotypes. Among memory CD4 cells we have now identified a distinct subset which is virtually negative for  $\alpha 4$ , in addition to the subsets which are  $\alpha 4\beta 7$  high and those which are  $\alpha 4\beta 1$  high. We have also performed 2D gel analysis of integrins on peripheral T cells. These studies demonstrate that naive and memory cells use the same complement of integrin chains, primarily  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 5$ ,  $\beta 1$ ,  $\beta 7$  and that the these chains are present on naive cells in roughly the same proportions as on memory cells. The most complex and informative patterns are observed with immunoprecipitations using  $\alpha 4$ , which reveals: 1) intact  $\alpha 140$  and cleaved  $\alpha 80$  and  $\alpha 70$ ; and 2) both  $\beta 1$  and  $\beta 7$ . Consistently, there is a greater relative abundance of  $\beta 7$  (relative to  $\beta 1$ ) in naive cells, suggesting a relatively greater contribution of  $\beta 7$  to the adhesive potential of naive cells.

Finally, we have expended substantial effort in organizing a component of the 5th International Workshop on Leukocyte Differentiation Antigens. This workshop is part of series of powerful international collaborative undertakings to systematically enumerate and characterize the cell surface molecules relevant to the immune system. We have conceived and organized a novel component of this workshop, which transcends the traditional organization of the workshop in lineage-specific subpanels of molecules (and antibodies). Our undertaking is to semi-quantitatively measure the expression of most known (and many poorly defined) cell surface molecules on cells of many lineages. We are doing this by assembling the most informative antibodies from the entire workshop (about 470!) and co-ordinating their analysis by about 30 motivated and competent flow cytometry labs who are studying cell types of particular interest to them. Our analysis is in progress. The excellence of the results is evident and this database of information is proving to be an extraordinarily powerful tool in our understanding of cell surface molecules and their functions.

Publications:

Tanaka Y, Albelda SM, Horgan KJ, van Seventer GA, Shimizu Y, Newman W, Hallam J, Newman PJ, Buck CA, Shaw S. CD31 expressed on distinctive T cell subsets is a preferential amplifier of  $\beta 1$  integrin-mediated adhesion. *J Exp Med* 1992;176:245-253.

Horgan KJ, Shaw S. Immunological memory. In: Roitt IM, Delves PJ, eds. *Encyclopedia of Immunology*. London: Sanders Scientific Publications 1992:1046-1049.

Horgan KJ, Tanaka Y, Shaw S. Post-thymic differentiation of CD4 T lymphocytes: Naive versus memory subsets and further specialization among memory cells. *Chem Immunol* 1992;54:72-102.

Horgan KJ, Luce GE, Tanaka Y, Schweighoffer T, Shimizu Y, Sharrow SO, Shaw S. Differential expression of VLA- $\alpha 1$  and VLA- $\beta 1$  discriminates multiple subsets of CD4+ CD45RO+ "memory" T cells. *J Immunol* 1992;149:4082-4087.

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van Seventer GA, Bonvini E, Yamada H, Conti A, Stringfellow S, June CH, Shaw S. Costimulation of T cell receptor/CD3-mediated activation of resting human CD4+ T-cells by LFA-1 ligand ICAM-1 involves prolonged inositol phospholipid hydrolysis and sustained increase of intracellular Ca<sup>++</sup> levels. *J Immunol* 1992; 149:3872-3880.

Tanaka Y, Shaw S. T cell adhesion cascades: General considerations and illustration with CD31. *Adv Exp Med Biol* 1992;323:157-162.

Radka SF, Webb D, Gerrard T, Van Seventer G, Shaw S, Del Prete G, Romagnani S. Immunologic stimuli and cell specificity of oncostatin M secretion in vitro. In: Romagnani S, Mosmann TR, Abbas AK, eds. *New advances in cytokines*. New York: Raven Press, 1992:137.

Tanaka Y, Adams DH, Shaw S. Proteoglycan on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol Today* 1993;14:111-114.

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

PROJECT NUMBER

Z01 CB 09263-11 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

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, PI):  
 P.A. Henkart Senior Investigator EIB, NCI

Others:	H. Nakamura	Visiting Fellow	EIB, NCI
	H. Park	Biologist	EIB, NCI
	M. Williams	IRTA Fellow	EIB, NCI
	R. Blumenthal	Microbiologist	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH  
 Experimental Immunology Branch

SECTION  
 Lymphocyte Cytotoxicity Section

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS: 4.2	PROFESSIONAL: 2.2	OTHER: 2.0
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CHECK APPROPRIATE BOX(ES)

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

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

In order to test the granule exocytosis model for lymphocyte cytotoxicity, we have examined the cytotoxic activity of the rat mucosal mast cell tumor line RBL after transfection with genes for cytotoxic lymphocyte granule components. We have constructed triple, double, and single RBL transfectants expressing cytolysin (cy) and the granule serine proteases granzyme A (gza) and granzyme B (gzb). RBL-cy transfectants show only modest cytotoxicity on tumor targets, with no accompanying target DNA degradation. While RBL-gza transfectants express gza at levels comparable to cloned CTL and secrete it in response to IgER cross-linking, they have no cytotoxic activity detectable. RBL-cy-gza transfectant clones showing good expression of both these granule components showed cytolytic activity comparable to RBL-cy on RBC targets, but were greater than 3x more lytic on three different tumor targets. This cytotoxicity is accompanied by target DNA fragmentation. To confirm that killer cell granzymes need to enter the target cell, we loaded target cells with the macromolecular protease inhibitor aprotinin by osmotic lysis of pinosomes. Compared to BSA-loaded targets or unloaded targets, aprotinin-targets were less susceptible to lysis and DNA breakdown by CTL and RBL transfectants expressing granzyme A. However, RBL transfectants expressing only cytolysin lysed BSA-loaded and aprotinin-loaded targets with equal efficiency. As a direct test of the ability of proteases to induce cytotoxicity when introduced into the cytoplasm of a target cell, we have "injected" various proteases into tumor cells using osmotic lysis of pinosomes. The endoproteases trypsin, chymotrypsin, and proteinase K were all found to lyse several different types of tumor cells in a dose dependent manner, as measured by <sup>51</sup>Cr release. This death was generally apoptotic by morphological criteria and DNA fragmentation.

## Project Description

Major Findings:

In order to test the hypothesis programmed cell death (PCD) in lymphocytes involves activation of an endogenous protease, we have tested the ability of protease inhibitors to block PCD in the T cell hybridoma 2B4. PCD and apoptotic characteristics induced by antibodies against the TcR or Thy-1 were blocked by the cysteine protease inhibitors E-64 and leupeptin, the calpain-selective inhibitor Acetyl-leu-leu-nor-meth, and the serine protease inhibitors DFP and PMSF. These inhibitors enhanced the TcR-induced IL-2 secretion, showing that they do not act by interfering with signal transduction. They enhanced PCD induced in 2B4 by corticosteroids, indicating that not all PCD pathways are inhibited. Thymocyte PCD induced by Ca ionophore, steroid, or TcR cross-linking were not inhibited by these protease inhibitors, while PCD in activated mature T cells induced by TcR cross-linking was blocked. We have tested the cysteine protease inhibitors for their ability to block PCD in peripheral blood lymphocytes from HIV<sup>+</sup> blood donors cultured for 2 days with pokeweed mitogen and superantigen. Under these conditions, little or no PCD was observed when normal or SLE donors were cultured, but significant PCD was seen in 58 of 95 HIV<sup>+</sup> donors. Blocking of this PCD was observed with members of the E-64 class (especially with the more hydrophobic compounds) as well as with leupeptin and the calpain inhibitor. These inhibitors were also tested for their ability to restore defective in vitro proliferative T cell responses to antigens and PHA. About 20% of the defective responses to influenza and 30-50% of the defective responses to alloantigen and PHA were restored to normal levels by these protease inhibitors. Control responses by normal donors were largely unaffected by these protease inhibitors, which were not mitogenic. These data suggest that a calpain-dependent pathway is involved in the TcR-triggered PCD pathway in both mature T lymphocytes and HIV<sup>+</sup> cells and that calpain inhibitors should be considered as candidates for therapy of HIV infection in humans.

Proposed course:

Other members of the E-64 family of cysteine protease inhibitors are being tested for their ability to block PCD in the T hybridoma and HIV<sup>+</sup> T cell systems. Other PCD systems are being tested for their ability to be blocked by protease inhibitors. Measurements of calpain activation after TcR cross-linking are being set up. Use of E-64d to inhibit TcR induced PCD in mature T cells in vivo is planned.

Publications:

None

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

PROJECT NUMBER  
 Z01 CB 09264-06 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Studies of T Lymphocytes function in transplantation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,  
 PI: G. M. Shearer Section Chief EIB, NCI  
 Other: M. Clerici Visiting Scientist EIB, NCI  
 R. Schulick Pratt Fellow EIB, NCI

COOPERATING UNITS (if any)  
 M. Weir, Dept. of Surgery, Univ. of Maryland School of Med., T.E. Starzl,  
 Dept. of Transplantation Surgery, Univ. of Pitts., J. Burdick, The Johns  
 Hopkins Univ. School of Med., John Miller, Fairfax Hospital, Fairfax, VA.

LAB/BRANCH  
 Experimental Immunology Branch

SECTION  
 Cell Mediated Immunity Section

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS: 2.5	PROFESSIONAL: 1.5	OTHER: 1.0
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CHECK APPROPRIATE BOX(ES)

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

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

Earlier studies from this laboratory demonstrated that the in vitro response of human T helper cells (Th) to HLA alloantigens is mediated by two distinct pathways for antigen presentation called the indirect and direct pathways, which respectively involve presentation of HLA antigens on self antigen presenting cells or direct presentation by the allogeneic stimulator cells. The laboratory further showed in renal transplant patients that the indirect pathway but not the direct pathway was correlated with kidney graft rejection.

We have now begun to study rejection of cardiac allografts in mice and humans. In the murine heteropic heart transplant model involving immune suppression by cyclosporin A, we found a correlation between rejection and an intact indirect pathway but not between rejection and an intact direct pathway. It appeared that rejection was mediated by host-anti-donor cytotoxic T lymphocytes (CTL). In patients who received a cardiac transplant, biopsy-determined lymphoid infiltration (the clinical test for rejection) was correlated with an intact indirect pathway but not the direct pathway of allorecognition. These findings suggest that (similar to the results of renal allografts) host antigen-presenting cells, rather than antigen presenting cells resident in the graft, are mainly responsible for cardiac allograft rejection. These results also suggest that our test may provide an immunologic assay that detects rejection of human heart transplants.

## Project Description

Major Findings:

We previously demonstrated that the rejection of human renal allografts was associated with the immunosuppressive drug-induced loss of the "indirect pathway" of T cell alloantigen recognition (the pathway that involves HLA alloantigen presentation via self or host antigen-presenting cells) but was not associated with the "direct pathway" of recognition (that involves direct recognition of HLA alloantigens on foreign antigen-presenting cells). Although we previously demonstrated that loss the indirect pathway was indicative of no short-term rejection, we also found that only 6/11 transplant recipients whom we predicted should reject their kidney allografts were actually undergoing rejection at the time of study. In a three-year follow-up study of the same patients, we found that: 0/5 of the patients who originally did not respond through either pathway retained their kidneys; 2/22 patients who had selectively lost their indirect pathways had lost renal function; and 9/10 of the original 11 patients whom we initially predicted would lose their renal allografts had lost their transplants three years after our initial analysis. These follow-up observations suggest that our test for renal allograft rejection is more sensitive than currently-used clinical tests, and is predictive for long-term loss of renal allografts, which is the major problem of kidney transplantation today.

Cardiac transplantation has a major problem in that there is no reliable and low-risk immunologic test for detecting the rejection of transplanted hearts. Current methods rely on expensive and risky cardiac biopsies. This laboratory has developed a two-pronged approach for investigating cardiac allograft rejection - one in the mouse, and the other in humans. Both are based on our experience with human renal allografts, and our finding that loss of the indirect pathway of allorecognition was sufficient for retention of human renal allografts.

In the murine model, adult BALB/c mice were ectopically transplanted beneath the skin of the ear with newborn heart from C57BL/6 mice. The mice were given different doses of cyclosporin A (CsA) at doses that would or would not selectively abrogate doses of CsA that left the indirect pathway of allorecognition. We found that doses of CsA that left the indirect pathway intact resulted in rejection, and was associated with host anti-donor cytotoxic T lymphocytes (CTL). In contrast, a dose of CsA that was sufficient to abolish the indirect pathway but not the direct pathway resulted in long-term retention of beating cardiac allografts. These results indicate rejection of murine cardiac allografts that involve recognition of both class I and II MHC determinants is mediated by the indirect but not the direct pathway of allorecognition.

Preliminary studies of human cardiac transplants suggest that the indirect but not the direct pathway of recognition will be important in the rejection of cardiac allografts. Thus, all biopsies that did not show extensive lymphoid infiltrates into the graft were associated with the loss of the indirect pathway. Approximately 50% of the patients whose in vitro blood lymphocyte assays predicted a potential rejection problem show evidence of lymphoid

infiltration. These latter data are similar to the early human renal allograft data see above, and may suggest that we can identify and predict patients who will have long-term problems with their heart transplants. These studies are continuing for the development of a reliable, non-invasive, and inexpensive test for the rejection of human cardiac allografts.

Due to the similarities between the selective immunosuppression of the CD4-sAPC pathway seen in immunosuppressed transplant patients and in asymptomatic HIV-infected individuals, 70% of this project is AIDS-related.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09265-11 EIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Analysis of the T Cell 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: L. Palmer Microbiologist EIB, NCI  
 M. Novick Summer Research Fellow EIB, NCI  
 C. Pucillo Visiting Fellow EIB, NCI  
 L. Selvey Visiting Fellow EIB, NCI  
 S. Sharrow Senior Investigator EIB, NCI

## COOPERATING UNITS (if any)

Cooperating units: BRMP, NCI  
 LTI, NCI  
 Villanova University

## 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

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

Exogenous retroviruses were analyzed for their influences on T cell repertoire. A defective murine leukemia virus which causes a mouse acquired immune deficiency syndrome (MAIDS) induced superantigen-like T cell activation in vitro. In vivo, this virus selectively activated and expanded CD4+ T cells expressing V $\beta$ 5, followed later in the course of infection by widespread immune deficiency in all T cells.

The effect of milk-borne MMTV on the T cell receptor (TCR) repertoire was analyzed. A previously uncharacterized tumorigenic milk-borne virus in BALB/c mice (the BALB/cV virus) was found to induce deletion of T cells expressing TCR V $\beta$ 2 in developing mice. This effect was MHC-dependent. The role of MHC class II molecules in susceptibility to MMTV infection was tested for the C3H MMTV. This milk-borne virus induced V $\beta$ 14 deletion only in strains of mice bearing natural or transgenic I-E class II major histocompatibility complex (MHC) product. Moreover, susceptibility to milk-borne virus as determined by assays of viral pp28 or LTR mRNA was also dependent upon I-E expression. These findings indicate that viral infection is dependent upon superantigenic stimulation of host lymphoid cells.

Although V $\beta$ -specific superantigenic effects are a useful model for the study of TCR selection, selection may more commonly be on the basis of receptor specificity determined by multiple TCR  $\alpha$  and  $\beta$  chain components. Analysis of the expression of specific TCR V $\alpha$ /V $\beta$  pairs has indicated that V $\alpha$ /V $\beta$  pairing is non-random and that strain-specific differences exist in patterns of V $\alpha$ /V $\beta$  expression, providing a new approach to the study of repertoire selection. T cell responses to endogenous superantigen were also shown to be influenced by V $\alpha$  as well as V $\beta$  TCR expression.

## Project Description

Major Findings:

## 1) Negative selection in generation of the T cell receptor repertoire.

Generation of the T cell receptor repertoire involves negative selection as a means of deleting those T cells which are potentially reactive to self determinants. It was found that significant strain-specific decreases in expression occur in at least 12 of the 22  $V\beta$  products and that each of these deletions is dominant in  $F_1$  mice, consistent with the conclusion that these deletions occur in the process of eliminating T cells with potential reactivity for self determinants. The role of the thymus in mediating TCR negative selection was analyzed by studying congenitally athymic nude mice. A comparison of T cell receptor  $V\beta$  expression in congenic pairs of normal and athymic mice indicated that the normal  $V\beta$  deletions associated with tolerance to self products did not occur in athymic mice. These results demonstrate that the thymus has a critical role in mediating self tolerance by negative selection.

2) Analysis of ligands mediating  $V\beta$ -specific negative selection.

Endogenous mouse  $V\beta$  deleting ligands have been mapped to endogenous MMTV genes by several laboratories. Segregation analysis of deletions of  $V\beta 5$ , 11, and 12 has demonstrated overlapping but non-identical influences of *mtv-8*, 9, and 11 proviruses. Use of a feral inbred strain which lacks MMTV proviruses supported the conclusion that only MMTV products act as endogenous  $V\beta$ -specific deleting ligands in mice. To determine whether species other than the mouse express ligands for  $V\beta$  deletion, bone marrow chimeras were constructed in which mixtures of mouse and rat bone marrow cells were injected into lethally irradiated mouse recipients. When mouse  $V\beta$  expression was analyzed in these chimeras, it was found that rat bone marrow-derived cells contributed in a rat strain-specific manner to the ligand for mouse  $V\beta$  deletion.

3) Selective expression of specific  $V\alpha/V\beta$  pairing.

With the exception of the  $V\beta$ -specific recognition of superantigens, T cell recognition of antigen is generally determined by multiple TCR  $\alpha$  and  $\beta$  chain segments. Selection of the T cell repertoire may therefore be detected by analysis, not of  $V\beta$  expression alone, but by expression of particular  $\alpha$  chain/ $\beta$  chain pairs. An analysis of expression of specific  $V\alpha/V\beta$  pairs by T cells indicated that  $V\alpha$ 's and  $V\beta$ 's are not randomly associated on peripheral T cells. Moreover, patterns of  $V\alpha/V\beta$  pairing differ between inbred mouse strains, suggesting that TCR repertoire selection influences this expression. Thus, the effect of conventional (non-superantigen) self antigens on the T cell repertoire may be amenable to investigation by this approach. In addition, when  $Mls^a$  (*mtv-7*)-specific T cells were selected by in vitro stimulation, it was found that  $V\alpha$  expression, in addition to the dominant influence of  $V\beta$  expression, plays a role in T cell specificity for endogenous *mtv* superantigen.

## 4) In vivo effects of exogenous retroviruses.

A defective murine leukemia virus (in combination with helper virus), has previously been described to produce an acquired immune deficiency state (MAIDS) in vivo. Products of this virus act as a superantigen in vitro to selectively stimulate  $V\beta 5$  and  $V\beta 11$ -bearing T cells. In vivo, at an early stage after viral infection, selective expansion and activation of  $V\beta 5^+CD4^+$  T cells was identified. Later in the course of infection, a deficiency was observed in early signal transduction through both TCR on T cells and sIg on B cells.

Milk-borne transmission of different strains of MMTV results in selective depletion of T cells expressing specific  $V\beta$  products. A newly characterized tumorigenic MMTV in BALB/c mice (BALB/cV virus) was found to induce specific deletion of  $V\beta 2$ -expressing T cells. Deletion requires the presence of appropriate MHC class II antigen. This virus has a unique LTR ORF sequence, correlating with its unique  $V\beta$  specificity. The role of superantigenic stimulation in susceptibility to milk-borne C3H MMTV infection was analyzed using mice that either do or do not express the class II MHC E $\alpha$  transgene. Deletion of  $V\beta 14$ -bearing T cells required the expression of an I-E product. Moreover, susceptibility to viral infection as measured by levels of viral pp28 or MMTV LTR mRNA was also dependent upon transgenic I-E expression. These results suggest that I-E-dependent superantigenic stimulation of  $V\beta 14$  T cells plays a facilitating role in host infection with milk-borne MMTV.

Proposed Course of Research:1) Analysis of ligands mediating  $V\beta$ -specific negative selection.

a) Identification of MTV product. Mapping and transfection studies have identified a role of the MTV LTR gene in  $V\beta$  deletion, but have not demonstrated whether or not the product of this gene is directly involved in T cell recognition or deletion. In collaboration with Dr. Janice Knepper (Villanova), antibodies specific for the BALB/cV LTR product will be made and used to study MTV expression in multiple tissues, including lymphoid and thymus populations. These antibodies will also be tested for effects on T cell responses to MTV superantigens. Class II-positive B cell lines will be transfected with the BALB/cV LTR or with mutated products and will be analyzed for superantigenic properties.

b) Mapping of additional  $V\beta$  ligands. The existence of  $V\beta$ -specific deleting ligands in species other than mouse, including human, will be studied by the technique of mixed bone marrow chimeras.

2) Selective TCR  $V\alpha/V\beta$  pairing.

It will be determined whether the observed non-random distribution of  $V\alpha/V\beta$  pairing reflects positive or negative immune selection of the T cell repertoire. Patterns of  $V\alpha/V\beta$  pairing will be determined in multiple inbred mouse strains. Where strain-specific patterns are observed, the genetic regulation of these patterns will be analyzed. The self ligands mediating any such selection will be identified by studies of genetic segregation.



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

PROJECT NUMBER

Z01 CB 09266-11 EIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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,

PI: R. J. Hodes Senior Investigator EIB, NCI

Others: K. Hathcock Chemist EIB, NCI

H. Hirano Visiting Fellow EIB, NCI

Q. Vos Visiting Fellow EIB, NCI

COOPERATING UNITS (if any)

Naval Medical Research Institute  
 Food and Drug Administration

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews

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

Stimulation of B cells with IL5 induces the appearance of a phenotypically novel B cell population which expresses high density of CD44 and low densities of B220 (CD45) and Ia. CD44 expressed by these cells mediates binding to the extracellular matrix material hyaluronic acid (HA), indicating a potential role for CD44 in trafficking of activated B cells in vivo. CD44 expressed on IL5-stimulated B cells migrates with a lower molecular weight than CD44 expressed by control B cells, reflecting differential glycosylation. No differences in CD44 mRNA isoform were apparent by PCR analysis. The B cell stimuli LPS and anti- $\delta$  do not induce CD44-dependent HA-binding activity. However, LPS-activated B cells demonstrate CD44-dependent HA binding rapidly after exposure to a unique CD44-specific mAb, suggesting that distinct functional states of the CD44 molecule exist reflecting differences in conformation or cytoskeletal association.

mAb was generated by immunizing rats with activated mouse B cells. One of these mAb (GL7) reacts with a subpopulation of activated B cells, as well as with activated T cells. GL7 precipitates a previously undescribed 29-31 KDa molecule from activated B cells. Another mAb (GL1) reacts with activated B cells. GL1 inhibits responses of CD4<sup>+</sup> T cells to activated B cells, suggesting that the target of GL1 may represent a costimulatory molecule for T cell activation.

To establish a system for the study of Th cell-B cell interaction at a single cell level, responses were generated using Ig transgenic B cells and cloned Th cells. Highly efficient hapten-specific responses were generated. The study of sera from these transgenic mice indicated that transgene-associated idiotype was expressed in association with endogenous Ig molecules.

## Project Description

Major Findings:

## 1) CD44 expression and B cell activation.

IL5 stimulation resulted in the appearance of a B cell subpopulation which is surface Ig bright, CD44 bright, B220 (CD45) dull, and Ia dull. This population was shown to contain nearly all of the proliferative and Ig secretory activity of IL5 activated B cells. In vivo activation of B cells by specific antigen challenge or by the induction of a stimulatory graft-versus-host reaction resulted in the appearance of a similar CD44<sup>hi</sup> population. Since evidence has suggested that CD44 can function as a cell adhesion molecule, with HA as one potential ligand, the ability of resting and activated B cells to bind to (HA) was assessed. It was found that IL5-activated B cells had a uniquely increased binding to HA, and this binding was inhibited by anti-CD44. These findings suggest that CD44 expression may represent a unique marker for B cells driven to proliferation and differentiation, and that CD44 itself may function as an adhesion molecule which is involved B cell trafficking in vivo. Other B cell activating stimuli such as LPS do not induce CD44-dependent HA-binding activity. However, LPS-activated B cells demonstrate CD44-dependent HA binding immediately after exposure to a unique CD44-specific mAb, suggesting that distinct functional states of the CD44 molecule exist, perhaps reflecting differences in conformation or cytoskeletal association.

<sup>35</sup>S-methionine metabolic labeling and <sup>125</sup>I surface labeling were used to characterize CD44 expression on activated or non-activated B cells. CD44 molecules expressed by IL5-activated B cells were found to migrate with a lower apparent molecular weight than CD44 isolated from control B cells. This difference in apparent molecular weight was eliminated by treatment with N-glycanase, suggesting that differential glycosylation of CD44 occurs in activated versus resting B cells.

PCR analysis was used to study the possible expression of multiple isoforms of CD44 by both lymphoid and non-lymphoid cells. Multiple isoforms of CD44 mRNA were identified which are generated by alternative splicing of 10 different exons. Patterns of CD44 expression were highly tissue-specific.

## 2) Expression of CD45 on resting and activated B cells.

T cells at various stages of activation and differentiation are known to express different isoforms of cell surface CD45, reflecting in part the differential splicing of several variable exons. In contrast, B cells have generally been characterized as expressing a uniformly high molecular weight isoform of CD45. Analysis of resting and activated B cells demonstrated that activation-specific changes are induced in the expression of serologically detected CD45 epitopes. These changes can be correlated with changes detected by immunoprecipitation. In addition, a polymerase chain reaction (PCR) analysis of CD45 mRNA expression indicates that unique changes in variable exon splicing are induced by specific B cell activation stimuli.

## 3) Identification of new B cell activation molecules.

In an effort to identify cell surface molecules uniquely expressed during activation of B cells, a series of mAb was generated by immunizing rats with activated mouse B cells. One of the resulting mAb (GL7) reacted by flow cytometry with a subpopulation of CD3-bright thymocytes, but at only a very low level with resting peripheral T or B cells. In contrast, GL7 reacted with con A-activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells and with a subpopulation (approximately 50%) of those B cells which were activated to size enlargement and increased Ia expression by stimuli including LPS or anti-Ig. This mAb precipitated a molecule of apparent molecular weight 29-31 kDa from either biosynthetically or surface labeled activated B cells. This appears to represent an activation molecule distinct from any previously described. Another mAb generated in this fashion (GL1) reacted with activated B but not T cells. GL1 inhibited responses of CD4<sup>+</sup> T cells to activated B cells, suggesting that the target of GL1 may represent a costimulatory molecule for T cell activation.

## 4) Mechanism of Th cell-B cell interaction.

A highly efficient system of specific Th cell-B cell interaction was established using Ig ( $\mu/k$ ) transgenic B cells, which uniformly express a hapten-specific Ig receptor, and cloned antigen-specific Th cells. This cell interaction results in the specific activation and differentiation of B cells to Ab secretion. In preparation for studying early activation events during Th-B cell activation, it was established that anti-receptor antibody induced vigorous intracellular [Ca<sup>++</sup>] responses in T or B cells as detected by flow cytometry.

## 5) Association of transgenic and endogenous Ig chains in Ig transgenic mice.

During characterization of Ig  $\mu/k$  transgenic mice, it was noted that a high proportion of serum Ig molecules of endogenous (non-transgenic) origin expressed the transgene idiotype. This observation could have resulted from the existence of mixed isotype Ig molecules, from extensive class switching by trans-rearrangement, or from a "network" influence on Ig expression. Analysis by ELISA, immunoabsorption, and gel filtration demonstrated that transgenic  $\mu$  chains associate in chimeric Ig molecules with endogenous  $\mu$  or  $\alpha$  chains produced by the same cell.

Proposed Course of Project:

## 1) CD44 expression and B cell activation.

The molecular basis for different functional properties of CD44, including hyaluronate (HA) binding by activated, CD44<sup>hi</sup> B cells will be studied. As described above, IL-5-activated B cells have both an increased quantitative level of cell surface CD44 and a qualitative change in CD44 reflected by differential behavior in gel analysis. The expression of alternatively spliced isoforms of CD44 will also be analyzed by PCR. mAbs will be generated by immunization with proteins corresponding to unique sequences of

alternatively expressed CD44 isoforms. Such mAb will be used to probe expression and functional role of CD44 isoforms. Attempts will also be made to study the role of CD44 using transgenic techniques including gene ablation by homologous recombination.

A role of CD44-mediated binding to extracellular matrix has been suggested in the in vivo trafficking of normal lymphoid cells and in the metastatic behavior of malignant cells. Preliminary experiments have demonstrated that IL5 stimulation of the murine B cell lymphoma BCL1 induces dramatically increased HA binding by these cells. The molecular basis for this will be studied. In addition, the effect of activation and altered HA binding upon in vivo trafficking of normal B cells and lymphoma cells will be analyzed.

#### 2) Expression of CD45 on resting and activated B cells.

PCR analysis will be used to characterize further the regulation of CD45 expression in resting and activated T and B cells. Changes in alternative splicing of CD45 mRNA have been induced in monoclonal B cell lines by activation. Preliminary data indicate that the expression of lower molecular weight CD45 mRNA, in which variable exons are spliced out, is dependent upon active protein synthesis.

#### 3) Identification of new B cell activation molecules.

Further functional characterization of the activation molecules recognized by GL7 and GL1 will be carried out. Collaborative studies have been initiated to identify the GL7 and GL1 target molecules by screening of an expression library constructed from activated B cell cDNA.

#### 4) Mechanism of Th cell-B cell interaction.

The antigen-specific interaction of Th cells and B cells will be analyzed by a digital imaging system in which intracellular  $[Ca^{++}]$  can be analyzed over time in individual Th/B cell conjugates. In this system, the ability of cell interactions to signal each of these populations will be analyzed. Subsequently, the role of antigen-specific and non-specific cell interaction molecules will be analyzed by testing the effects of mAb specific for such molecules.

#### Publications:

Vos Q, Hodes RJ. Immunoglobulin mu, kappa transgenic mice express transgenic idiotype on endogenously rearranged IgM and IgA molecules by secretion of chimeric molecules. *J Exp Med* 1992;176:951-961.

Kenny JJ, Sieckmann DG, Freter C, Hodes R, Hathcock K, Longo DL. Modulation of signal transduction in phosphocholine-specific B cells from  $\mu$ k transgenic mice. *Curr Top Microbiol Immunol* 1992;182:95-103.

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

PROJECT NUMBER  
 Z01 CB 09267-11 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Cellular Immune Function in AIDS and in Primary Immune Deficiencies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,  
 PI: G. M. Shearer Section Chief EIB, NCI  
 Others: M. Clerici Visiting Scientist EIB, NCI  
 D. Lucey Cancer Expert EIB, NCI  
 J. Berzofsky Senior Investigator EIB, NCI  
 P. Pizzo Chief EIB, NCI  
 Y. Yarchoan Senior Investigator EIB, NCI  
 S. Broder Director EIB, NCI

COOPERATING UNITS (if any)  
 Craig Hendrix, Matthew Dolan, HIV Unit, Lackland AFB, TX; A. Landay, Rush Med. Ctr., Chicago, IL, J. Sever, Childrens' Natl. Med. Ctr., Washington, D.C, Robert Coffmanm DNAX, Palo Alto, CA.

LAB/BRANCH  
 Experimental Immunology Branch

SECTION  
 Cell Mediated Immunity Section

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:  
 3.9

PROFESSIONAL:  
 2.6

OTHER:  
 1.3

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

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

The spectrum of T helper cell (Th) defects observed in the peripheral blood leukocytes (PBL) of asymptomatic HIV-seropositive (HIV<sup>+</sup>) individuals has now been found to be predictive for time to AIDS diagnosis and time to death. The spectrum of Th defects appears to be attributable to a reversal from a predominance of Th1 over Th2 function to a predominance of Th2 over Th1 function. Thus it maybe that Th1 function is protective whereas Th2 is not.

The numbers of individuals and of cohorts of individuals who exhibit potent Th activity against HIV antigens but who are seronegative continue to grow and expand. At present, among HIV-exposed but seronegative individuals the percent of HIV-specific Th responsive individuals are: gay men, 63%; intravenous drug users, 45%; accidentally-exposed health care workers, 75%; surviving exposed hemophiliacs, 19%; and newborns of HIV-infected mothers, 35%. The majority of these individuals remains seronegative on follow-up, although a few are PCR<sup>+</sup> for HIV DNA.

The studies summarized above raised the possibility that cellular immunity, mediated by Th1 cells, is protective, but humoral immunity, mediated by Th2 cells is not. If correct, these findings would indicate that AIDS vaccine development should be directed to augment cellular rather than humoral immunity.

## Project Description

Major Findings:

The laboratory continues to obtain new cohorts as well as additional individuals within the cohorts already under study of HIV-exposed, seronegative individuals whose T cells generate strong helper responses, and in some cases cytotoxic T lymphocyte activity, to HIV specific antigens. The two new cohorts that have been added during the past year are long-term surviving hemophiliacs who received HIV-contaminated Factor VIII until 1985-86, and female sexual partners of HIV-infected men. Thus from 19% to 75% of seronegative individuals from every category of HIV-exposed individuals exhibit this pattern of strong T cell reactivity without evidence of antibody production. Follow-up study of a limited number of these individuals indicate that: 1) approximately 15% of the gay men go on to seroconvert and develop AIDS; 2) none of the health care workers seroconvert, but lose the Th responses to HIV within a year of exposure (unlike gay men and drug abusers); 3) 15% of the newborns of HIV<sup>+</sup> mothers became infected, but none of these infections were among the infants who exhibited HIV-specific Th immunity at birth. Three of six gay men who were T cell responsive but seronegative tested positive by PCR, indicating that they had integrated HIV genetic material. The results outlined above raised the possibility that cell-mediated but not antibody-mediated immunity is protective against HIV infection and/or progression of AIDS.

A longitudinal study of 355 of the asymptomatic HIV-seropositive (HIV<sup>+</sup>) individuals whom we had "staged" into four Th functional categories based on their responses to recall antigens, allo-antigens, and PHA, indicated that our staging criteria +/++, -/+ and -/- were predictive for time to diagnosis of AIDS and time to death. For example, only 8% of +/+, individuals progressed to AIDS in 3 years. 24% of -/+ and 48% of -/-/+. A similar (but lower %) pattern was seen for patients who died in the subsequent three years. These findings indicates that our functional "staging" of HIV<sup>+</sup> individuals is predictive for two relevant events in progression to AIDS.

Our finding that T helper cell function assessed by proliferation and IL-2 production is lost prior to the appearance of AIDS symptoms has now been complemented with the findings that: 1) IFN- $\gamma$  is also reduced; 2) IL-4 is increased and then decreased; 3) IL-10 is subsequently increased; and 4) in vitro addition of anti-IL-4 and anti-IL-10 antibodies reverse the T helper cell functional loss, as does the addition of IL-12. These findings strongly support our hypothesis that the loss of T helper function in the progression toward AIDS is accompanied by a switch from a predominant Th1 to a predominant Th2 condition, and that a Th1 response is protective against HIV infection and/or progression to AIDS, whereas a predominant Th2 response is not.

As a model for studying cellular and humoral immunity in AIDS, macaques were exposed intrarectally to a spectrum of SIV ranging from  $10^{-3}$  to  $10^3$  infectious doses. The high dose exposed macaques made antibody and weak or no cellular immunity, became infected and developed AIDS-like symptoms. The lower dose exposed macaques generated strong cellular immunity, but not antibody response, and none developed an AIDS-like illness. These findings raise the possibility of low dose immunization for the selective activation of potentially protective cellular immunity against HIV.

Because the envelope of HIV-1 gp120 contains 3-dimension conformational regions similar to non-polymorphic regions of human HLA class I and II, we have tested whether allo-stimulated peripheral blood leukocytes (PBL) from healthy HIV-uninfected individuals would lyse autologous targets pulsed with synthetic peptides corresponding to these regions. PBL from approximately 60% of uninfected individuals exhibited such reactivity, but did not lyse peptides unrelated to these regions.

A high proportion of AIDS patients on growth hormone therapy exhibit 4-to-10-fold increases in T helper cell immune function, even to HIV synthetic peptides. 100% of this research is AIDS-related.

Publications:

Clerici M, Giogi JV, Gudeman VK, Chou CC, Zack JA, Nishanian PG, Dudley JP, Berzofsky JA, Shearer GM. Specific T-helper immunity to HIV-1 envelope peptides in seronegative individuals with recent exposure to HIV-1. *J Infect Dis* 1992;165:1012-1019.

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Sher A, Gazzinelli RT, Oswald I, Clerici M, Kullberg M, Pearce EJ, Berzofsky JA, Mosmann TR, James SL, Morse HC, Shearer GM. Role of T cell derived cytokines in the down-regulation of immune responses in parasitic and retroviral infection. *Immunol Revs* 1992;127:183-204.

Clerici M, Shearer GM. The use of in vitro T cell immune function to monitor the course of HIV infection. In G Janossy, B Autran, F Miedema (Eds): *Immunodeficiency in HIV infection and AIDS*. Karger Basel 1992:pp 64-75.

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Clerici M, Roilides E, Via CS, Pizzo P, Shearer GM. A factor from CD8 cells of human immunodeficiency virus (HIV)-infected patients suppresses HLA self-restricted T helper cell responses. *Proc Natl Acad Sci USA* 1992;166:723-730.

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Clerici M, Shearer GM. A TH1-TH2 switch is a critical step in the etiology of HIV infection. *Immunology Today* 1993;14:107-111.

Clerici M, Hakim FT, Venzon DJ, Blatt S, Hendrix CW, Wynn TA, Shearer GM. Changes in TH1 and TH2 cytokine production in asymptomatic, HIV-seropositive individuals. *J Clin Invest* 1993;91:759-765.

Salk J, Bretscher P, Salk PL, Clerici M, Shearer GM. A strategy for prophylactic vaccination against HIV. *Science* 1993;260:1740-1742.



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

PROJECT NUMBER

Z01 CB 09268-06 EIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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, PI: Alfred Singer Chief EIB, NCI

Others: David Wiest Special Volunteer EIB, NCI  
 Kelly Kearse IRTA Fellow EIB, NCI  
 Patricia Benveniste Special Volunteer EIB, NCI  
 Ken Katz IRTA Fellow EIB, NCI

COOPERATING UNITS (if any)

LP, NCI; CBMB, NICHD; Naval Medical Research Institute, Univ. of Washington  
 Harvard School of Public Health

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4.75

PROFESSIONAL:

3.75

OTHER:

1.0

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(a) Human       (b) Human tissues       (c) Neither  
 (al) Minors  
 (a2) Interviews

B

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

We have found that T cell receptor (TCR) expression and function in developing thymocytes is actively regulated by CD4-mediated signals generated by the interaction of CD4 with Ia<sup>+</sup> thymic epithelium. CD4 molecules on the surface of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes are engaged in situ by Ia<sup>+</sup> thymic epithelium and transduce intracellular signals that result in: (i) low TCR expression, (ii) tyrosine phosphorylation of TCR-zeta chains, and (iii) inability of TCR cross-linking to induce intracellular calcium flux. Release from these intra-thymically generated inhibitory CD4 signals results in increased TCR expression, dephosphorylation of TCR-zeta chains, and improved TCR signaling. Further, we have found that the molecular basis for low TCR expression in developing CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is a high rate of degradation of newly synthesized TCR components, and that CD4 mediated signals regulate the TCR degradation rate in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes via tyrosine kinase p56 lck which was shown to be preferentially associated with CD4, rather than CD8, in immature thymocytes. It was also shown that membrane bound protein tyrosine phosphatase CD45, a regulator of lck activity, can regulate intra-thymic T cell differentiation suggesting a role in thymocyte development for ligands of CD45.

## Project Description

Major Findings:

To examine the role of CD4 signals 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 caused a 3-5 fold increase in surface expression of TcR on immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. It was also found that physical separation of immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from Ia<sup>+</sup> thymic epithelium caused the thymocytes to spontaneously increase their expression of TCR *in vitro*. Furthermore, CD4 signals, induced by multivalent cross-linking of anti-CD4 mAb, mimicked the presence of thymic epithelium by inhibiting TCR expression. The mechanism of TCR inhibition in immature double positive thymocytes was the retention and degradation in the Endoplasmic Reticulum of newly synthesized and assembled TCR complexes, a process that was regulated by CD4-mediated signals.

Because CD4 is associated with the tyrosine kinase p56 lck, we examined the phosphorylation status of TCR-zeta, a tyrosine kinase substrate, in developing thymocytes. Consistent with the presence of a tonic CD4 signal in immature double positive thymocytes, we found that TCR-zeta was already phosphorylated in immature thymocytes resident in the thymus, but that they spontaneously dephosphorylated upon being separated from thymic epithelium. We next investigated the role of CD4-associated lck molecules in regulating TCR expression in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and the activation of CD4-associated lck molecules by CD4 engagement. It was found that CD4 regulation of TCR expression in immature thymocytes is dependent upon a CD4-associated tyrosine kinase, presumably lck, and that activation of lck in these cells results from engagement of surface CD4 molecules, but not CD8 surface molecules. The competence of CD4 to activate lck in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes was due to a relatively large fraction of surface CD4 molecules (25-50%) associated with intracellular lck molecules. In contrast only 2% of surface CD8 molecules were associated with lck. The amount of lck associated with CD4 in immature thymocytes was also found to be inversely related to the extent of CD4 engagement by MHC class II molecules in the thymus. These studies demonstrate a novel function for an intracellular tyrosine kinase in the regulation of TCR distribution and expression in immature thymocytes.

Because lck was found to regulate TCR expression in immature thymocytes, we next investigated the possibility that the membrane bound protein tyrosine phosphatase CD45, known to regulate lck activity, might play a role in thymocyte differentiation. Using both *in vivo* and *in vitro* treatments, it was found that antibody engagement of CD45 on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes: 1) enhances lck tyrosine kinase activity; 2) inhibits TCR expression; and 3) inhibits differentiation of immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes into mature single positive T cells. These studies demonstrate that the ability of immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to undergo positive selection can be regulated by CD45 and suggest a potentially important regulatory role for intrathymic ligands that are capable of engaging CD45 on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.

We also examined the ability of surface TCR complexes on immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to transduce signals leading to intracellular calcium mobilization. We found that surface TCR complexes on "uninduced" CD4<sup>+</sup>CD8<sup>+</sup> thymocytes that had not yet escaped from CD4-mediated inhibition signaled very poorly as measured by calcium mobilization, whereas TCR on "induced" CD4<sup>+</sup>CD8<sup>+</sup> thymocytes that had been released from CD4-mediated inhibition mobilized calcium as well as mature T cells. The relative inability of TCR on uninduced CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to signal for intracellular calcium mobilization was a function of both their low receptor number and the phosphorylation state of their TCR-zeta chains.

The consequences of *in vivo* TCR-transduced signals, such as those which are thought to occur during TCR repertoire selection, were assessed by measuring intracellular calcium concentrations in freshly prepared thymocytes not subjected to any further stimulation. It was found that approximately 3% of normal thymocytes had significant elevations of [Ca<sup>2+</sup>], even though these cells had not been intentionally stimulated with exogenous antigen. The majority of these cells with elevated calcium levels were CD4<sup>+</sup>CD8<sup>+</sup> immature thymocytes, the subset in which most thymic selection events are thought to occur. In order to examine the relationship between [Ca<sup>2+</sup>] and intrathymic signaling in freshly prepared thymocytes, anti-H-Y TCR $\alpha\beta$  transgenic mice were used. This allowed comparison of developing thymocytes with identical TCR specificity in a negatively selecting *in vivo* environment (male thymus expressing H-2D<sup>b</sup> plus male antigen H-Y) versus a positively selecting *in vivo* environment (female thymus expressing the MHC-restricting element H-2D<sup>b</sup>). It was found that *in situ* thymocytes with elevated calcium levels were increased in male, but female animals. This increase was due to cells expressing the transgenic TCR and was organ-specific in that male T cells with elevated [Ca<sup>2+</sup>] were found only in the thymus and not in the spleen of individual animals. Unlike thymocytes from normal animals, those transgenic male thymocytes with elevated calcium levels were of the CD4<sup>-</sup>CD8<sup>-</sup> phenotype, consistent with the interpretation that these cells have recently undergone and survived negative selection. These studies indicate that endogenous ligands do stimulate developing thymocytes to mobilize intracellular Ca<sup>2+</sup> *in vivo*, and that such intrathymic signaling events are evident in thymocytes expressing self-reactive TCR.

In order to pursue more detailed biochemical analyses of TCR complexes, a method for physical separation of immature and mature murine TCR complexes was developed. This methodology, based on processing on N-linked carbohydrate side chains, uses wheat germ agglutinin (WGA) affinity matrices to separate TCR complexes which have reached the trans Golgi compartment of the cell from those that have not. The technique is rapid, sensitive, maintains integrity of the assembled TCR complexes, and provides an additional approach for the study of TCR assemble and intracellular transport.

Publications:

Nakayama T, Ueda Y, Yamada H, Shores EW, Singer A, June CH. In Vivo calcium elevations in thymocytes with TCR that are specific for self ligands. Science 257:96-99, 1992.

Anderson SJ, Abraham KM, Nakayama T, Singer A, Perlmutter RM. Inhibition of T-cell receptor  $\beta$ -chain gene rearrangement by overexpression of the non-receptor protein tyrosine kinase p56<sup>lck</sup>. EMBO J. 11:4877-4886, 1992

Kearse KP, Wiest DL, Singer A. Subcellular localization of T cell receptor complexes containing tyrosine-phosphorylated zeta proteins in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Proc. Natl. Acad. Sci. (USA). 90:2438-2442, 1993.

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

PROJECT NUMBER

Z01 CB 09273-06 EIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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, PI: Alfred Singer Chief EIB, NCI

Others: Elizabeth Shores IRTA Fellow EIB, NCI  
 Joseph Roberts Guest Researcher EIB, NCI  
 Yousuke Takahama Visiting Fellow EIB, NCI  
 Jennifer Punt IRTA Fellow EIB, NCI  
 Harumi Suzuki Visiting Fellow EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

5.75

PROFESSIONAL:

4.75

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

(a) Human  (b) Human tissues  (c) Neither  
 (al) Minors  
 (a2) Interviews

B

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

The intra-thymic differentiation of functionally and phenotypically distinct T cell subsets, as well as their interaction with thymic epithelium, has been examined. Studies on thymocytes from genetically defective scid mice have suggested that TCR+ cells play a critical role in promoting the entry of thymocytes into the CD4/CD8 differentiation pathway as well as in promoting the maturation and organization of thymic medullary epithelium. The role of TCR expression by the differentiating thymocytes themselves was studied by using scid mice possessing an already rearranged TCRV $\beta$  transgene which allows development of mature T cells. It was found that T cells at different stages of development expressed structurally distinct TCR complexes and that full differentiation into mature single-positive T cells requires surface expression of fully assembled TCR complexes. Early thymocyte differentiation was studied by *in vivo* and *in vitro* analysis of requirements for the transition of precursor thymocytes to CD4+CD8+ double positive cells. It was found that: a) negative selection can occur before the CD4+CD8+ stage of differentiation; b) cross-linking of TCR molecules on precursor thymocytes blocks their further differentiation by elimination of messenger RNA's encoding the co-receptor molecules CD4 and CD8 and the recombination activating genes 1 and 2; c) TCR-induced post-transcriptional regulation was specific for selective messenger RNAs, required protein synthesis, and was developmentally regulated; and d) the rate at which CD4+CD8+ thymocytes are generated in the thymus is controlled by TGF- $\beta$ .

## 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 the requirements for TCR expression in T cell differentiation. It was found that Thyl<sup>+</sup> thymocytes from most scid mice contain only CD4<sup>-</sup>CD8<sup>-</sup> (double negative) TCR<sup>-</sup> cells which are similar to double negative cells from the thymi of normal mice. Introduction of TCR<sup>+</sup> cells into the thymi of scid mice promoted differentiation of scid thymocytes into CD4/CD8 expressing cells which themselves remained TCR<sup>-</sup>. Immunohistologic examination of the thymic stroma in scid mice demonstrated while thymic medullary epithelium failed to organize and mature in the absence of TCR<sup>+</sup> cells, the introduction of TCR<sup>+</sup> cells into the scid thymus induced the normal maturation and organization of thymic medullary epithelium. Thus, these studies emphasize the importance of reciprocal interactions between thymocytes and thymic stroma in T cell and thymus development.

The requirements for TCR assembly and expression by the differentiating thymocytes themselves were studied using TCRV $\beta$ 8-transgenic scid mice which express a rearranged TCR $\beta$  transgene, but are impaired in their ability to rearrange and express endogenous TCR $\alpha$  genes. It was found that TCR $\beta$ -transgenic mice contained not only immature double positive thymocytes, but also possessed mature CD4<sup>+</sup> and CD8<sup>+</sup> single positive T cells in both the thymus and periphery. Biochemical analyses of TCR expression demonstrated that immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes expressed surface TCR $\beta$  chains without a clonotypic TCR partner chain and largely without associated CD3 components. In contrast, mature single-positive T cells in these transgenic animals expressed fully assembled surface TCR complexes which consisted of disulfide-linked heterodimers pairing transgenic TCR $\beta$  chains with endogenous TCR $\alpha$  chains and which were associated with CD3 components. Further, the surface TCR complexes on these mature cells were competent to transduce TCR-mediated proliferative signals. These studies show that T cells at different stages of development in TCRV $\beta$ 8-transgenic scid mice express structurally distinct surface TCR complexes and that the developmental stage reached by individual T cells in these animals is related to the structural nature of the surface TCR complexes expressed by those cells. These results are important to our understanding of T cell differentiation because they suggest that successful T cell differentiation normally requires surface expression of fully assembled TCR complexes.

During thymic differentiation, T cells progress through an ordered sequence of developmental stages best characterized by expression of the co-receptor molecules CD4 and CD8. The first cells to enter the CD4/CD8 developmental pathway are CD4<sup>-</sup>CD8<sup>10+</sup> thymocytes which are the immediate precursors of CD4<sup>+</sup>CD8<sup>+</sup> double positive thymocytes, a transition which occurs spontaneously in *in vitro* suspension culture. The requirements for this *in vitro* differentiation of murine CD4<sup>-</sup>CD8<sup>10</sup> precursor thymocytes into CD4<sup>+</sup>CD8<sup>+</sup> cells were studied. It was found that this differentiation step could be inhibited by TCR crosslinking or by the drug phorbol myristate acetate (PMA), a potent activator of protein kinase C (PKC). This TCR-mediated inhibition of differentiation was not mediated by inhibition of transcription, but rather by elimination of mRNAs encoding two distinct families of molecules involved in the development of early thymocytes: 1) the co-receptor molecules CD4 and CD8 which mediate crucial cellular interactions; and 2) the recombination activating genes (RAG) 1 and 2 which are required for rearrangement of TCR gene loci. TCR induced elimination of these mRNAs was specific, required protein synthesis and was itself developmentally regulated. These studies identify a post-transcriptional mechanism that is influenced by TCR signals and that regulates early thymocyte development.

Because *in vitro* antibody-mediated TCR signals could inhibit the transition of CD4<sup>-</sup>CD8<sup>10</sup> precursor thymocytes to CD4<sup>+</sup>CD8<sup>+</sup> double positive cells, we next studied the possibility that antigen-mediated TCR signalling could affect negative selection in precursor thymocytes. The phenotype and *in vitro* differentiation capacity of fetal thymocytes that expressed transgenic TCRs with defined antigen specificity, D<sup>b</sup> + H-Y. It was found that in a negatively selecting male thymus, CD4<sup>-</sup>CD8<sup>10</sup> precursor thymocytes expressing transgenic TCR to male antigen were developmentally arrested and failed to become CD4<sup>+</sup>CD8<sup>+</sup>. This antigen-mediated negative selection of precursor thymocytes could also be demonstrated *in vitro*. The *in vitro* differentiation of Tg-TCR CD4<sup>+</sup>CD8<sup>10</sup> thymocytes was inhibited by antigen-presenting cells (APCs) from male H-2<sup>b</sup> nude mice that express the antigenic ligand for the TCR transgene, but was not inhibited by APCs from female H-2<sup>b</sup> nude mice. These studies demonstrate that intra-thymic negative selection of developing T cells can occur prior to the CD4<sup>+</sup>CD8<sup>+</sup> stage of differentiation.

The non-TCR mediated requirements for progression of developing thymocytes along the CD4/CD8 developmental pathway were also analyzed. It was found that progression through one cell cycle is necessary for differentiation of CD4<sup>-</sup>CD8<sup>10</sup> precursor thymocytes into CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, and that progression through the cell cycle is specifically regulated by interaction with cortical thymic epithelial cells. The regulatory ligands expressed by cortical thymic epithelial cells were identified as transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) and TGF- $\beta$ 2. These studies identify a novel regulatory mechanism that acts on developing precursor thymocytes independently of TCR, and that is mediated by cortical thymic epithelial cells.

Publications:

Roberts JL, Abe R, Shores EW, Singer A. Expression of Mls determinants in mice exhibiting the severe combined immunodeficiency (*scid*) mutation or X-linked immunodeficiency (*xid*) defect. *J Immunol.* 149:1577-1582, 1992.

Stein PH, Rees MA, Singer A. Reconstitution of (BALB/c x B6) $F_1$  normal mice with stem cells and thymus from Non-Obese Diabetic (NOD) mice results in autoimmune insulinitis of the normal hosts' pancreas. *Transplantation* 53:1347-1352, 1992

Takahama Y, Shores EW, Singer A. Negative selection of precursor thymocytes before their differentiation into  $CD4^+CD8^+$  cells. *Science* 258:653-656, 1992.

Takahama Y, Singer A. Post-transcriptional regulation of early T cell development by T cell receptor signals. *Science* 258:1456-1472, 1992.

Shores EW, Nakayama T, Wiest DL, Sharrow SO, Singer A. Structurally distinct T cell receptor complexes on developmentally distinct T cell populations in severe combined immunodeficiency mice expressing a  $TCR\beta$  transgene. *J. Immunol.* 150:1263-1275, 1993.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09279-08 EIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Regulation of Expression of MHC Class I Genes

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

P. I.: Dinah Singer Section Chief EIB, NCI

Others: Jocelyn Weissman Chemist EIB, NCI

Kevin Howcroft Staff Fellow EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

Molecular Regulation Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## 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.)

MHC class I genes encoding transplantation antigens are ubiquitously expressed, although their level of expression varies among tissues. Analysis of the 5' flanking DNA sequence of a swine class I gene has demonstrated that this region contains a series of negative and positive regulatory elements. One of these elements, consisting of overlapping negative and positive regulatory elements, is a regulatory domain responsible for establishing tissue-specific levels of MHC class I gene expression. Introduction into transgenic mice of a series of nested deletion mutants which differ in the extent of the regulatory domain, reveals that the enhancer activity predominates in lymphoid tissues. The tissue-specific domain forms distinct enhancer and silencer associated complexes with cellular trans acting factors. Enhancer binding activity is present in extracts from various cell types, independent of levels of class I expression. In contrast, the level of silencer binding is inversely proportional to the level of class I gene expression. Thus, class I genes are found to be negatively regulated. Biochemical characterization of the regulatory factors has demonstrated that each factor consists of at least two distinct components, one of which appears to be common to both factors. Both the silencer and enhancer factors are redox-sensitive. The enhancer factor complex is approximately 30kD. The enhancer unique subunit is also glycosylated. The silencer factor complex is approximately 95kD.

An additional negative regulatory element has been identified which the proto-oncogene, c-jun, binds as a homo or heterodimer, and acts as a specific negative regulator. These observations suggest that class I expression is actively regulated during cell activation, and also suggest a mechanism whereby cells transformed by oncogenic variants of c-jun could decrease class I expression.

## Project Description

Major Findings:

Expression of individual MHC class I genes is actively regulated: large differences in the levels of class I gene expression are observed among tissues. Thus, expression is high in lymphoid tissues, but low in other tissues such as kidney and liver. However, even among the lymphoid tissues, there are distinct differences in the level of expression, such that B cells express twice as much class I as do T cells. In earlier studies, we demonstrated that introduction of one of the swine class I genes, PDI, into a transgenic mouse resulted in its regulated expression, in a pattern indistinguishable from that observed *in situ* in the pig. These studies indicated that regulatory sequences responsible for establishing normal patterns of expression were contained within the transgene. To further define the regulation of this class I gene, we have undertaken a detailed analysis of the 1.1 kb of 5' DNA sequences flanking the PDI promoter, and have identified a series of positive and negative regulatory elements. Using a series of 5' deletion mutants, as well as discrete DNA segments, ligated to the reporter gene CAT, we have identified the canonical transcriptional promoter, the interferon response element, and an array of positive and negative regulatory elements.

One of these elements maps between -700 and -800 bp upstream of transcriptional initiation. This element is a complex regulatory element, consisting of two overlapping functional elements: a silencer and an enhancer. Together these elements comprise a tissue-specific regulatory domain, which establishes tissue-specific levels of class I gene expression. The enhancer is comprised of an interrupted, inverted repeat, whereas the silencer consists of two discontinuous 10 bp binding sites, spaced by 10 bp. Enhancer binding factors are constitutively expressed in all tissues examined, including tissues which do not express class I. In contrast, the level of silencer binding factor is inversely proportional to the level of class I expression. Thus, in tissues where class I expression is low, high levels of silencer binding factors are observed. That this tissue-specific regulatory domain functions *in vivo* has been demonstrated through the analysis of transgenic mice generated using a variety of deletion constructs either containing or deleted of the domain. The enhancer was observed to function in lymphoid tissues, but not in non-lymphoid tissues. The patterns of expression of the transgene in non-lymphoid tissues was consistent with the activity of the silencer. Biochemical analysis of the enhancer and silencer binding factors has revealed that each is composed of two subunits; it is likely that one of the subunits is shared between the two factors. None of the subunits is capable of binding DNA independently. Both silencer and enhancer factors are redox sensitive, as evidenced by the observation that treatment with either diamide or NEM results in loss of activity. The enhancer factor is a 30 kD complex, one subunit of which is glycosylated. The silencer factor is a 95 kD complex.

A second regulatory element maps between -418 and -447 bp upstream of transcriptional initiation. This element functions as a negative regulator of MHC class I gene expression. In the presence of this element, transcription from downstream promoters, either homologous or heterologous, is reduced 3-4 fold. Contained within this region is a TRE-like DNA sequence element. TRE elements are the recognition sites for the transcription factor, AP1. We have

now shown that c-jun binds to this site and functions as a transcriptional silencer of class I expression. Thus, cotransfection of a c-jun reporter gene with a class I promoter construct containing the TRE-like element results in a 3-4 fold decrease in promoter activity. Stable introduction of the c-jun expression vector into L cells causes a 10-15 fold decrease in endogenous class I gene expression. DNase foot-printing studies confirm the binding of c-jun to the TRE-like element. In vivo, this element functions as a silencer since in all tissues in transgenic mice there is greater transgene expression in the absence of the element than in its presence.

Publications:

None

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

PROJECT NUMBER

Z01 CB 09281-07 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Receptor Mediated T and B Cell Activation

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

PI:	R. J. Hodes	Senior Investigator	EIB, NCI
Others:	K. S. Hathcock	Chemist	EIB, NCI
	M. Okajima	Biotechnology Fellow	EIB, NCI
	L. Selvey	Visiting Fellow	EIB, NCI

COOPERATING UNITS (if any)

Naval Medical Research Institute

LAB/BRANCH  
 Experimental Immunology Branch

SECTION  
 Immune Regulation Section

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

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

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

The effect of prior activation history on subsequent responses of cloned T helper 1 (Th1) cells to TCR-mediated stimuli was examined. Th1 cells were maintained by stimulation with IL2 alone or by stimulation with specific antigen and APC in addition to IL2. Cells carried under both conditions proliferated equivalently in response to anti-CD3 antibody. However, anti-CD3 induced strong phosphatidyl inositol (PI) hydrolysis and increased  $[Ca^{++}]_i$  only in cells that had been maintained by stimulation with IL2 alone; cells that had been stimulated with specific antigen + APC gave neither PI nor  $Ca^{++}$  responses. The signaling pathways utilized by Th1 cells were thus influenced by prior stimulation through the TCR.

Receptor-mediated activation was analyzed in T and B lymphocytes from normal mice and from mice infected with the MAIDS-inducing defective murine leukemia virus. Several weeks after viral infection, the proliferative responses of T and B cells to cross-linking of TCR and sIg respectively were significantly reduced despite the expression of normal surface levels of these receptors by most T and B cells. To analyze early signaling events in these cells,  $[Ca2+]_i$  was measured in response to surface receptor cross-linking. The  $[Ca2+]_i$  responses of both T and B cells from MAIDS-infected mice were decreased. B cell responses to sIg cross-linking were further analyzed by examining protein tyrosine phosphorylation induced by sIg cross-linking. It was found that after virus infection, there was a progressive loss of selected tyrosine phosphorylation events with conservation of other events. The response defect in B cells from MAIDS mice is thus reflected in selected alterations of tyrosine phosphorylation in response to sIg signaling.

## Project Description

Major Findings:

## 1) Activation of Naive T and B Cells.

Signal transduction was analyzed in T and B lymphocytes from normal mice and from mice infected with the MAIDS retrovirus. Several weeks after viral infection, most T and B cells expressed normal surface densities of TCR and sIg respectively. There was, however, a significant decrease in proliferative responses of both T and B cells to receptor cross-linking. The  $[Ca^{2+}]_i$  responses of T and B cells to the same stimuli were similarly decreased. The response of B cells to sIg cross-linking was further studied by analyzing tyrosine phosphorylation. B cells from normal mice exhibited substantial increases in tyrosine phosphorylation of a number of proteins upon stimulation. B cells from MAIDS mice were progressively defective in phosphorylation of several of these proteins, but maintained normal phosphorylation of other proteins.

## 2) Activation of Cloned T Cells.

The Th1 clone AE7.6 is stimulated to proliferate by immobilized anti-CD3 antibody in the absence of accessory cells or exogenous lymphokines. The influence of prior stimulation upon subsequent responsiveness of these cloned cells was analyzed by carrying clone AE7.6 in vitro either by stimulation with IL2 alone or by stimulation with specific antigen and APC in addition to IL2. Lines maintained by these two protocols gave equivalent proliferative responses to anti-CD3 stimulation. However, marked differences were seen in the induction of second messengers by this stimulation. Cells carried in IL2 alone generated substantial PI hydrolysis as well as increased  $[Ca^{++}]_i$  in response to anti-CD3. In contrast, cells that had been previously stimulated with specific antigen and APC, and then allowed to "rest" gave markedly reduced PI and  $Ca^{++}$  responses. The signaling pathways activated in these T cells are thus strongly influenced by the recent activation history of these cells.

## 3) Signal transduction in T cell responses to endogenous superantigen.

The nature of signal transduction events induced in cloned and heterogeneous populations of peripheral T cells by encounter with endogenous superantigens was evaluated. A flow cytometric system was established which allows study of conjugate formation between individual superantigen-specific T cells and APC bearing endogenous superantigen, and which simultaneously measures  $[Ca^{++}]_i$  changes in the T cells involved in these conjugates. In contrast to several recent reports, both PI hydrolysis and increased  $[Ca^{++}]_i$  were induced in peripheral T cells responding to Mls<sup>a</sup>. Peripheral T cells from mice which express Mls<sup>a</sup> were unresponsive to self Mls<sup>a</sup> as determined by the lack of proliferative or  $[Ca^{++}]_i$  responses, consistent with tolerance to self antigens. In contrast, thymocytes from the same mice, although failing to proliferate in response to Mls<sup>a</sup> stimulators, did form specific cell conjugates with these stimulators and exhibited strong  $[Ca^{++}]_i$  responses. These results indicate that immature thymocytes, prior to negative selection, respond specifically to self superantigen. This response may reflect the signals involved in negative selection of self-reactive T cells during intrathymic differentiation.

Proposed Course of Project:

## 1) Activation of Naive T Cells.

The T cell populations which are defined by patterns of CD45 isoform expression will be analyzed to determine their functional characteristics, including their responsiveness to TCR stimuli and the effect of CD45 cross-linking on these responses. The relationships among these populations during intra-thymic and post-thymic T cell differentiation will be studied by approaches including cell fractionation and in vitro activation. The regulation of CD45 isoforms during activation will be evaluated using polymerase chain reaction to identify alternatively spliced CD45 mRNA, as well as by biochemical and serologic analysis.

## 2) Activation of cloned T Cells.

The molecular basis underlying differences in  $Ca^{++}$  and PI responses in cloned T cells will be investigated. The effect of cell permeabilization upon PI hydrolysis in response to TCR cross-linking will be studied in order to probe for possible roles of inhibitory intracellular mediators. cDNA probes for different phospholipase C genes will be used to study the expression of these genes in cloned T cells giving high or suppressed  $Ca^{++}$  and PI responses. These studies will be extended to a panel of Th1 and Th2 clones.

## 3) Signal transduction in T cell responses to endogenous superantigen.

The ability to measure specific conjugate formation and  $[Ca^{++}]_i$  responses by individual T cells will be applied to studies of immature T cell populations during intrathymic development, to anergic T cells generated in transgenic and bone marrow chimeric animals, and to retrovirus-infected immunodeficient mice. If early  $[Ca^{++}]_i$  responses are abnormal in any of these instances, the nature of the proximal defect will be analyzed by structural assessment of the TCR as well as by measurement of early phosphorylation events. If normal  $[Ca^{++}]_i$  responses are observed, later activation events will be analyzed to determine the nature of response defects in these cells.

## 4) Analysis of activation-specific cell surface molecules.

mAbs have been generated by immunization of rats with activated mouse B cells. Several of these mAbs were found to be specific for known molecules including CD44, CD45, MHC class II, and sIg. In addition, several mAbs appear to identify previously uncharacterized molecules expressed by activated B and T lymphocytes. One of these mAbs inhibits co-stimulus-dependent T cell activation and appears to identify a newly identified ligand for CTLA4. The mechanism of T cell response inhibition by this mAb will be analyzed, and its ligand will be identified by expression cloning.

Publications:

Abe R, Ishida Y, Yui K, Katsumata M, Chused TM. T cell-receptor mediated recognition of self ligand induces signaling in immature thymocytes prior to negative selection. J Exp Med 176:459-468, 1992.

Hathcock KS, Hirano H, Murakami S, Hodes RJ.: Analysis of CD45 expression by B cells stimulated with IL5, anti-Ig, or LPS: Expression of different CD45 isoforms by subpopulations of activated B cells. *J Immunol* 1992;149:2286-2294.

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

PROJECT NUMBER

Z01 CB 09282-07 EIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Murine and Human Autoimmunity

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

PI:	G. M. Shearer	Section Chief	EIB, NCI
Others:	B. Bermas	Medical Staff Fellow	EIB, NCI
	E. Mozes	Guest Worker	EIB, NCI

COOPERATING UNITS (if any)

H. Peck, Department of Rheumatology, University of Maryland School of Medicine, Baltimore, MD.; C. S. Via, Department of Medicine, University of Maryland School of Medicine, Baltimore, MD.

LAB/BRANCH

Experimental Immunology Branch

SECTION

Cell Mediated Immunity Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.4

PROFESSIONAL:

1.9

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

This laboratory has established that the pattern of T helper cell (Th) dysfunction seen in asymptomatic, HIV-seropositive (HIV<sup>+</sup>) is also detected in patients with systemic lupus erythematosus (SLE). Thus, there are certain similarities between the immune dysregulation of SLE patients and HIV<sup>+</sup> individuals. To investigate other possible similarities, we have tested the sera of SLE patients and mice with SLE-like diseases, and have found evidence of antibodies that recognize the gp120 of HIV, as well as certain synthetic peptides of HIV envelope. These results provide further evidence of similar immunodysregulatory events in SLE patients and individuals infected with HIV.



## Project Description

Major Findings:

Certain parallels have been observed between autoimmune diseases and AIDS, including the production of autoantibodies in both conditions. This laboratory has demonstrated that the spectrum of Th defects in HIV<sup>+</sup> individuals can also be seen in outpatients who have SLE. Thus, approximately 50% of SLE outpatients are unable to respond to one or more antigenic stimuli in vitro.

To test for additional parallels between SLE and HIV infection, the sera of mice and of humans with SLE were tested by ELISA for antibodies reactive with the gp120 of HIV-1 envelope. Both the MRL-lpr/lpr strain that naturally acquires SLE, and the BALB/c strain which develops a SLE-like condition when injected with the 16/6 idiotype produced antibodies reactive with gp120. 43% of SLE patients produced antibodies reactive with gp120 compared with 12% of healthy controls and 14% of patients with other autoimmune diseases. Both SLE mice and patients produced antibodies that reacted with three of six tested synthetic peptides of HIV-1 envelope, and the sera of both species reacted with the same three peptides. Removal of anti-DNA antibodies did not deplete the sera of antibodies to gp120, indicating that these are distinct populations of antibodies. These findings indicate that the immunodysregulatory events between SLE and HIV infection occur at the antibody as well as at the T cell level, and include antibodies specific for HIV-1 envelope.

Publications:

Sugihara S, Fujiwara H, Shearer GM. Autoimmune thyroiditis induced in mice depleted of particular T cell subsets: The characterization of thyroid-inducing T cell lines and clones derived from thyroid lesions. *J Immunol* 1993;150:683-694.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09285-07 EIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Responses of MHC Class I Genes to Exogenous Stimuli

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

P.I. Dinah Singer Section Chief EIB, NCI

Others: Lisa Palmer IRTA EIB, NCI  
 Leonard Kohn Senior Investigator EIB, NCI  
 Motoyasu Saji Visiting Fellow EIB, NCI  
 Cesidio Juliani Guest Worker EIB, NCI  
 Georgio Napolitano Guest Worker EIB, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

Molecular Regulation Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## 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.)

MHC class I genes are affected by a variety of exogenous stimuli which can either increase or decrease levels of expression. Although agents such as TNF and interferon are well known modulators of class I genes, many other factors also alter expression. We have observed that the thyroid stimulating hormone (TSH) specifically reduces transcription of endogenous class I genes in cultured thryocytes. Thryocytes normally express MHC class I, as does a rat thryocyte cell line, FRTL-5. TSH treatment of FRTL-5 cells decreases transcription of both TSH receptor and class I genes. This down-regulation is cAMP mediated and TSH receptor dependent. The TSH responsive element has been located within 68 bp upstream of the class I promoter, to a region which contains only canonical promoter elements. Analysis of cell extracts from normal and TSH-treated thryocytes reveals TSH-mediated differences in the factors binding to a DNA sequence 3' to the TATAA box.

Other agents are capable of modulating class I expression. Among them, insulin, hydrocortisone, and serum act as a negative regulators of class I, whereas thyroid hormone is a positive regulator. Their sites of action are distinct from those of TSH. The DNA element responsive to serum maps to a constitutive negative regulatory element, RE-105. Analysis of RE-105 does not reveal a recognizable serum response element (SRE). However, an AP1-like binding site occurs within this region.

## Project Description

Major Findings:

MHC class I genes are regulated both by homeostatic and non-homeostatic regulatory mechanisms. Among the known exogenous, non-homeostatic regulators are interferon and TNF. Recent studies from our laboratory have identified a number of other agents that dynamically modulate class I gene expression. Among these is the hormone, TSH. To examine the effects of TSH and other hormones on class I expression, we have studied a rat thyrocyte line, FRTL-5, which responds in culture to TSH by increasing synthesis of thyroid peroxidase, thyroglobulin, and iodide uptake. Concomitantly, TSH receptor expression declines. Thyrocytes normally express relatively low levels of class I, as does the FRTL-5 line. However, following TSH treatment, FRTL-5 expression of class I decreases even further. This decrease is evident both at the cell surface and in steady-state levels of RNA. Transcription of class I genes is reduced to about one-half to one-third the basal level following TSH treatment of the cells. This response to TSH depends on the TSH receptor (TSHr), since a variant cell line which does not express receptor does not modulate class I response to TSH. Reintroduction of TSHr by transfection of the TSHr gene restores the response. Although TSH triggers a small change in intracellular calcium, its major effect is to increase intracellular cAMP levels. Directly increasing intracellular cAMP in FRTL-5 cells by treatment with forskolin, cholera toxin or 8-bromocAMP mimics the effect of TSH. We have identified the upstream flanking sequences of the class I promoter which are responsive to the TSH effect.

Using a series of 5' deletion mutants, derived from the promoter proximal region of a class I gene, ligated to a reporter gene, were introduced into the FRTL-5 line cultured in either the presence or absence of TSH. By this approach, it was demonstrated that only 68 bp of DNA sequences 5' of the transcription initiation site are required for TSH-mediated down-regulation. The only known DNA sequence elements contained within this region are the CAT and TATA boxes. Analysis by gel shift of cell extracts in combination with a series of overlapping double-stranded oligonucleotides spanning this 68 bp region, reveals that a TSH-responsive element is located 3' to the TATA box. Although the 68 bp segment is sufficient to confer TSH-sensitivity, additional regulatory elements appear to contribute to the overall response. The TSH-sensitive changes in DNA binding complexes that occur within the 68 bp region are influenced by upstream elements, which independently function as transcriptional regulatory elements.

Class I expression is also regulated by a variety of other agents, including insulin, hydrocortisone and serum. All three of these agents reduce transcription of class I sequences. Using the same series of 5' deletion mutants used to map the TSH-responsive element, the sites of action of these agents have been mapped also. They are all at sites distinct from that of the TSH element. In particular, the hydrocortisone response element maps to a region between -135 and -209 and is detected by changes in complex formation in a gel shift assay in response to hydrocortisone.

Publications:

Saji M, Moriarty J, Ban T, Singer D, Kohn L. MHC Class I Gene Expression in Rat Thyroid Cells is Regulated by Hormones, Methimazole, and Iodide, as well as Interferon. J Clin Endo Metab 1992;75:871-878.

Kohn LD, Kosugi S, Ban T, Saji M, Ikuyama S, Giuliani C, Hidaka A, Shimura H, Akamizu T, Tahara K, Moriarty J, Singer D. Molecular Basis for the Autoreactivity Against Thyroid Stimulating Hormone Receptor. International Immunological Reviews 1992;9:135-165.

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

PROJECT NUMBER

Z01 CB 09287-06 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Marrow Graft Rejection in Allogeneic Bone Marrow Transplantation

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

PI:	Ronald E. Gress	Senior Investigator	EIB, NCI
Others:	Kazuhiro Kurasawa	Visiting Fellow	EIB, NCI
	Dan Fowler	Clinical Associate	MB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH  
 Experimental Immunology Branch

SECTION  
 Transplantation Immunology Section

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:  
 1.0

PROFESSIONAL:  
 1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human       (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. Because host CTL in isolation could reject donor marrow grafts, effects on engraftment by (1) cell populations able to suppress host CTL responses, and (2) the administration of anti-CD3 monoclonal antibody in vivo, which by previous work had been shown to suppress CTL function, were studied. Cells with a specific type of suppressor activity, termed veto cells, which might suppress host rejection responses, have been reported to be present in marrow. The ability of IL-2 to enhance the activity of veto suppressor cell populations remaining in marrow after T cell depletion was investigated in vitro and in vivo. It was found that the incubation of T cell depleted marrow with IL-2 significantly increased veto activity as assessed by in vitro assays and also enhanced engraftment of MHC-mismatched, T cell depleted marrow in vivo, and that veto cells exerted their effect by clonal deletion of precursor CTL. Such clonal elimination involved participation by precursor CTL as well as veto cells. In further studies of engraftment of T cell depleted allogeneic marrow, host mice were treated with anti-CD3 monoclonal antibody. Marked enhancement of engraftment was observed; this effect on engraftment was enduring and due to suppression of host T cell function and to the release of multiple cytokines associated with in vivo activation of T cells by anti-CD3 antibody.

## Project Description

Major Findings:

The purpose of these studies was to directly assess the ability of murine CTL to reject allogeneic marrow grafts and to evaluate the effect that suppression of CTL function in vivo might have on the engraftment of T cell depleted, MHC-mismatched marrow. It was found that CTL clones isolated from 650 cGy sublethally irradiated mice, which had successfully rejected allogeneic marrow, suppressed MHC mismatched marrow graft proliferation (measured by  $^{125}\text{IUdR}$  uptake) when adoptively transferred into a 1025 cGy lethally irradiated B6 host if, and only if, the grafted marrow cells expressed MHC determinants for which the individual clone had cytotoxic specificity. These investigations therefore demonstrated that (1) a cloned CTL population is sufficient to reject an allogeneic marrow graft, and (2) the mechanism by which these marrow grafts are rejected is specific for MHC gene products expressed by the donor marrow corresponding to the cytotoxic specificity of the CTL clone. Parallel studies were undertaken with similar findings utilizing human CD8+ CTL with specificity for murine MHC encoded gene products.

Cells with a specific type of suppressor activity, termed veto cells, have been reported to be present in marrow. These cells suppress those precursor CTL with specificity for antigens expressed on the surface of the veto cells. The ability of IL-2 to enhance the activity of veto suppressor cell populations remaining in marrow after T cell depletion was investigated in vitro; it was found that the incubation of T cell depleted marrow with IL-2 significantly increased veto activity. Therefore, the possibility that marrow rejection by host CTL might be suppressed by IL-2 treatment of donor marrow was evaluated. Such treatment was associated with enhanced engraftment if IL-2 was given to the host animal in addition to the treated marrow. The mechanism by which veto cells suppress CTL responses is not known. Two barriers to the study of the mechanism has been unreliable suppression of CTL responses by putative veto cell populations, and the low frequency of precursor CTL in the responder population, making it technically difficult to differentiate death of precursor CTL from induction of anergy. The first difficulty was overcome by incubation of the suppressor cell population with IL-2. Studies showed an inhibition of veto activity by antisera with specificity for cytolytic granules, indicating that lysis of precursor CTL with clonal elimination, rather than induction of clonal anergy, may be the likely mechanism for the suppression of CTL responses by IL-2 enhanced veto cells. Studies with transgenic mice, in which responder T cell populations contain precursor CTL with a defined antigen specificity at high frequency, directly demonstrated that the mechanism by which veto cell activity mediates suppression of CTL responses is by clonal deletion of precursor CTL. Additional studies utilizing CSA have shown that the precursor CTL, in interacting with veto cells, play an active role in their own clonal elimination.

With the demonstration that CTL are sufficient to effect marrow graft rejection, studies were undertaken to evaluate the possible effects of anti-CD3 treatment on marrow engraftment in a mouse model. The antibody used was 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. The intact antibody results in detectable T cell activation in vivo while the F(ab')<sub>2</sub> from of the antibody does not. T cell immunosuppression is pronounced at one week after administration of the intact antibody. It was found, however, that in vivo treatment with anti-CD3 administered three to seven days before infusion of bone marrow did not fully 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 allogeneic marrow graft. Studies also demonstrated that administration of intact anti-CD3 to mice resulted 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). Because this activation also resulted in secretion of colony stimulating factors (CSF) detectable in the serum and was associated with extramedullary hematopoiesis in the spleen, the effect of anti-CD3 antibody administration at the time of allogeneic marrow infusion was evaluated. The injection of anti-CD3 monoclonal antibody with the donor marrow resulted in extensive allogeneic chimerism. Non-activating, anti-T cell monoclonal antibodies also facilitated engraftment, but resulted in lesser degrees of chimerism. Similar findings occurred in studies in subhuman primates. In the mouse, incubation of T cell depleted allogeneic marrow in the supernatant of spleen cells incubated with anti-CD3 antibody in vitro also resulted in enhancement of engraftment in the presence of, but not in the absence of, host T cell suppression. Therefore, the enhancement of marrow engraftment by in vivo administration of anti-CD3 monoclonal antibody appears to be due to both suppression of host T cell function and the presence of growth factors. Identification of specific factors which are present in the supernatants of spleen cells exposed to anti-CD3 monoclonal antibody, and which promote engraftment of T cell depleted, MHC-disparate marrow indicates that multiple factors are involved. In addition, it was found that even sublethal levels of irradiation resulted in enhancement of engraftment of donor marrow in the presence of exogenous hematopoietic growth factors. This differential ability of irradiated host and donor hematopoietic precursor cells to compete for growth factors expands current understanding of the concept of marrow space.

#### Publications:

- Hiruma K, Gress RE. Cyclosporine A and peripheral tolerance: Inhibition of veto cell-mediated clonal deletion of postthymic precursor cytotoxic T lymphocytes. *J Immunol* 1992;149:1539-1547.
- Hirsch F, Poncet P, Freeman S, Gress RE, Sachs DH, Druet P, Hirsch R. Antifection: A new method for targeted gene transfection. *Trans Proc* 1993;25:138-139.

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

PROJECT NUMBER

Z01 CB 09288-06 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 T Cell Function in T Cell Depleted Bone Marrow Transplantation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,  
 PI: R. E. Gress Senior Investigator EIB, NCI  
 Others: C. Mackall Clinical Associate EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH  
 Experimental Immunology Branch

SECTION  
 Transplantation Immunology Branch

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:  
 1.0

PROFESSIONAL:  
 1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B, D

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

The elimination of T cells from marrow is of interest both in allogeneic and autologous marrow transplantation -- as a means of preventing graft versus host disease in allogeneic marrow transplantation and as a means of eliminating or purging malignant cells expressing T cell surface markers from marrow in treating T cell neoplasms by autologous marrow transplantation. We developed approaches for depleting normal and malignant T cell marrow populations from marrow; these approaches were then used in clinical protocols assessing the feasibility of utilizing allogenic HLA-mismatched, T cell depleted marrow and autologous marrow purged of malignant T cells in the treatment of aggressive hematolymphopoietic malignancies. Preclinical studies in rhesus monkeys demonstrated that CD4+ T cell reconstitution and development of in vivo T cell immunocompetence correlated with the number of T cells infused in the marrow 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 conclusion has been confirmed in murine studies in which three T cell progenitor pools have been identified which contribute to final T cell repopulation following marrow transplantation. The functional capacities of these regenerated T cell populations is also of interest. The human T helper cell response to xenogenic MHC encoded antigens expressed by stimulating murine cell populations has been studied and found to be of special use in the assessment of human T helper cell function in that this primary response requires reprocessing of the stimulating murine antigens and presentation in association with human Class II gene products. The requirement for reprocessing of murine antigen and presentation by responder-type cells (rather than murine stimulating cells) was found to be due to a lack both of murine antigen presenting cell activation and responder T cell activation.



## Project Description

Major Findings:

The primary approach taken in these studies of T cell depletion of human marrow has been elimination of T cell populations by antibody plus complement and elutriation. Initial studies with antibody and complement established optimal conditions 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. Antibodies were selected for the ability to detect antigens expressed by malignant as well as normal T cells: CD7, CD2 and CD5. A fourth antibody was added to this combination which is specific for a CD unassigned T cell determinant. This determinant is expressed by cells of hematopoietic origin, is confined to T cells, and is concordant in its expression with CD5 and CD3. Immunoprecipitation with the antibody demonstrates a 92 KD molecule under non-reducing conditions and a predominate 45 KD band under reducing conditions. Comparisons of expression of the determinant defined by this antibody with those defined by antibodies of known specificity on a series of T cell lines, including a line deficient in the expression of T cell receptor, failed to identify the determinant defined by this antibody.

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 T cell depletion are insufficient in sensitivity to detect clinically relevant numbers of residual cells. A limiting dilution assay was therefore developed based on the clonogenic potential of peripheral human T cells; 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 used to monitor T cell depletion of human marrow. The processing of human marrow for clinical use has now been adapted to a closed, semi-automated system, which includes elutriation followed by treatment with antibody plus complement. The development of reagents and techniques for the removal of cells expressing T cell surface markers from marrow has resulted in clinical trials in both allogeneic marrow (HLA mismatched) and autologous marrow (with removal of malignant T cells) transplantation. With respect to the former, severe GvHD has been prevented with preservation of engraftment. With respect to the latter, the first stage of a phase I study has been completed with definition of a new preparative regimen for the eradication of neoplastic disease in vivo and the development of methods for peripheral marrow progenitor harvest and grafting.

To study T cell repertoire generation following T cell depleted marrow transplantation, we characterized the reconstitution of T cell populations in rhesus monkeys which had received untreated or extensively T cell depleted autologous bone marrow following myeloablative, lethal radiation. By phenotypic analysis, CD2+/CD8+/CD28- 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 slow rate of recovery of CD4+ cells was comparable to the rate of recovery for CD8+/CD28+ 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 or 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. 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 (with subsequent restriction specificity for host MHC antigens) is of central importance to considerations of MHC mismatched BMT in man. Subsequent studies which we have undertaken in murine models have indicated that, in the setting of marrow transplantation with T cell containing marrow, T cell reconstitution involves three precursor-containing pools, the marrow, residual T cells of the host, and infused T cells. Each gives rise to distinct progeny. In circumstances of limited thymic function, the latter pool dominates T cell reconstitution. T cell surface markers have been identified which allow determination of the presence of a functioning thymic-dependent pathway of T cell generation. These findings in murine models have been observed also in the populations of T cells regenerating in patients who have received intensive chemotherapy. The indication from primate studies that mature T cell populations might play a role in T cell reconstitution following marrow transplantation is therefore verified by these murine and human studies.

In addition to studies of the generation of T cell populations following marrow transplantation, the functional responses of the resultant T cell populations to antigenic stimulation is of interest. In particular, responses of T helper cells is important because T helper cell dysfunction has been observed in autologous as well as allogeneic marrow transplantation. One limitation in the study of human T helper cell function has been the scarcity of approaches to evaluate primary, MHC restricted T helper cell responses in man. Studies of human anti-mouse CTL responses indicate that a CD4+ helper pathway functions in the generation of CTL responses and that there exists a dependence on the presence of human antigen presenting cells. Of six xenogeneic responses evaluated, only the human antimurine response was dependent on human antigen presenting cells for CTL generation. The defective human CD4+T helper cell-murine stimulator cell interaction could be bypassed by the addition of exogenous IL-2 indicating that the dependence was at the level of a human helper T cell - stimulator cell interaction and did not reflect requirements at the level of the precursor CTL. The function of the responder antigen presenting cells involved in the human antimurine cytotoxic response was inhibited by chloroquine, suggesting a requirement for antigen processing. Effective presentation of murine stimulating antigen by human

antigen presenting cells was completely blocked by anti-human Ia antibody, indicating that the antigen is presented to human T helper cells in association with human class II molecules. These results were consistent with an Ia-dependent recognition of processed murine antigen by human T cells and represents an approach for assessing human T helper cell function and MHC restriction in a primary T cell response. Additional studies indicated that the defective interaction of human helper cells and murine antigen presenting cells involved a lack of activation of the latter. Currently, the inability to effectively interact seems to involve defects at the level of antigen presenting cell activation and initial T cell activation. Late events in T cell activation appear to be intact as activated murine APC effectively stimulate human T cells to produce IL-2.

Publications:

Read EJ, O'Shaughnessy JA, Yu MY, Cottler-Fx M, Denikoff AM, Cowan KH, Gress RE. Flow cytometric quantitation of circulating hematopoietic progenitor cells in breast cancer patients on chemotherapy. In Gross, S., Gee, A., and Worthington-White, D.A. (eds.): Bone Marrow Purging and Processing. Wiley-Liss. New York 1992:523-530.

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

PROJECT NUMBER

Z01 CB 09289-04 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Single Chain Antibodies and Related Projects.

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

PI:	D. M. Segal	Section Chief	EIB, NCI
Others:	I. Kurucz	Visiting Fellow	EIB, NCI
	A. George	Special Volunteer	EIB, NCI
	C. Jost	Visiting Fellow	EIB, NCI

COOPERATING UNITS (if any)

Creative Biomolecules, Inc. Hopkinton, MA  
James S. Houston, PI

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immune Targeting Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

3.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B, D

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

1. Several plasmids containing inserts encoding single chain Fv (sFv)-myc-peptide fusion proteins have been produced by genetic engineering techniques. Two of these, anti-DNP sFv-myc and anti human transferrin receptor-myc, have been produced in bacteria, and give active protein capable of binding to cell surfaces and to target lysis in the presence of an anti-myc x anti-CD3 bispecific antibody. Other sFv constructs have been produced in COS cells. These are secreted directly into the medium as active proteins capable of binding to cell surface antigens. Fusion proteins of anti-CD3 and other sFv's are being constructed to see if a single chain bispecific antibody can be produced.

2. An sFv of the 2B4 murine T cell receptor has been produced. The 2B4 sFv is monomeric in aqueous solution and binds three mAbs that bind to the parental TCR. It specifically blocks antigen presentation to 2B4 cells at  $\mu\text{M}$  concentrations, and specifically blocks superantigen mediated activation of T cells.

## Project Description

Major FindingssFv antibodies

The binding sites of antibodies reside in their variable regions, each consisting of one light chain variable domain ( $V_L$ ) and one heavy chain variable domain ( $V_H$ ). The  $V_H$  and  $V_L$  domains interact non-covalently, forming a globular region that contains a large antigen binding surface. Isolated V regions are known as Fv fragments. A single polypeptide chain construct with all of the binding activity of the native Fab fragment can be prepared by linking the C terminus of the  $V_L$  to the N terminus of the  $V_H$ , or vice versa, with a polypeptide spacer of at least 12 residues. This construct is known as a single chain Fv (sFv), and it has been prepared by recombinant DNA technology. The purpose of this project is to use sFvs to study protein folding and to construct various sFv fusion proteins that could be used to target cytotoxic cells.

By using appropriate oligonucleotide primers, constructs encoding sFv proteins were PCR amplified from hybridoma cDNAs and cloned into various plasmids. We have generated several sFv clones including 2C11 (anti-mouse CD3), OKT9 (anti-human transferrin receptor) and U7.6 (anti-DNP). OKT9 and U7.6 sFvs each contained a C-terminal myc-peptide and were expressed in bacteria using a periplasmic secretion system. Resultant protein was insoluble, but gained activity upon solubilization in denaturing solvents, and dialysis against non-denaturing medium. For each sFv, we obtained several mg of monomeric protein from a liter of bacterial culture. These sFv-myc fusion proteins were able to bind specifically to cells expressing the appropriate antigen (as tested by FACS analysis using FITC anti-myc), and were able to target lysis of cells in the presence of an anti-CD3 x anti-myc bispecific antibody. This was the first indication that sFv proteins could be used as targeting agents, and could provide a means by which sFvs, with their excellent tissue penetration, could be used to target cytotoxic cells against unwanted cells in vivo.

We have also produced sFvs in COS cells, in order to use the mammalian protein folding machinery to produce active protein. We have found that COS cells that are transfected with sFv containing plasmids are able to secrete active protein into the medium. We have used COS 7 cells to produce 2C11-myc, OKT9-myc, and U7.6-myc fusion proteins. Pulse-chase experiments of OKT9 sFv (which contains a glycosylation site) indicate that this sFv rapidly enters the ER following synthesis, and then slowly enters the Golgi, and finally is secreted into the medium. Secreted sFv from all three constructs, binds specifically to cells expressing the appropriate antigen, as judged by FACS analysis. These results show that sFv proteins, which differ in structure from intact native IgG, can nevertheless be processed normally by mammalian cells. COS secretion provides a method for rapidly testing sFv constructs for activity, and could be especially useful for testing more complex fusion proteins, for example bispecific sFvs.

### T Cell Receptor sFv

A single chain Fv from the 2B4 T cell receptor (TCR) was constructed by linking the C terminus of  $V_{\alpha}$  with the N terminus of  $V_{\beta}$  through a (gly<sub>4</sub>ser)<sub>3</sub> polypeptide linker. Protein was produced in *E. coli* as inclusion bodies, and after in vitro refolding, stable monomeric protein, was obtained. This protein bound three different monoclonal antibodies that are specific for conformationally dependent determinants on the native 2B4 TCR. The bacterially-produced 2B4 TCR-sFv blocks antigen presentation to 2B4 cells (and a T cell clone with similar specificity) by APC, but does not block presentation to other clones with different specificity. It also blocks superantigen mediated stimulation of 2B4 cells. Blockage of antigen presentation occurs in the  $\mu\text{M}$  range, presumably by binding to the I-E<sup>k</sup> peptide complex on the APC, and competing with the cell bound TCR. The concentration dependence of inhibition suggest that the binding constant of the construct to the MHC-peptide complex is in the  $10^6 \text{ M}^{-1}$  range. In summary, the TCR-sFv provides sufficient material to examine TCR-antigen interactions.

### Current and future plans.

#### sFv-antibodies

We plan to work mainly with COS cell expression and to proceed in two directions. First we will attempt to link the 2C11 sFv to the OKT9 sFv to see if we can make a single-chain bispecific targeting molecule. This will involve inserting a spacer in between the two molecules, and we plan to try poly proline, thioredoxin and ubiquitin. The latter two are small globular proteins with high solubility and stability that could both separate the two sFvs and improve the stability and solubility of the construct. We will isolate the construct from culture supernatant and test it for the ability to target lysis of cells such as HUT102 that express high amounts of huTfr. In addition we will make an sFv construct of W6/32, an antibody against human class I molecules that also cross reacts on COS 7 cells. We have already found that insertion of a KDEL sequence on the C-terminus of OKT9-sFv causes it to be retained in the ER. We will see whether a KDEL sequence on W6/32 causes it to be retained, and in so doing down regulates Class I expression on COS cells. This could provide a valuable tool for removing, or strongly down regulating cells surface receptors in general.

#### sFv-TCR

Our immediate plans are to use the 2B4 construct to probe TCR/peptide-Class II interactions. Although we have demonstrated that our current construct has TCR activity, it does suffer from solubility and folding problems. Preliminary experiments have indicated that fusion of a solubilizing peptide onto the 2B4 TCR-sFv greatly enhances its solubility and ease of folding. We will therefore try to improve on the current construct by engineering solubilizing and stabilizing fragments to the N and C termini. In addition, we have seen some indication that the TCR-sFv binds directly to APC, by using FACS analysis. We will try to improve on that assay with the new construct.

We will then try to get a good estimate of the affinity and specificity with which 2B4 binds antigen, both in the direct and indirect assays. Following that we will begin mutating residues to see which portions of the TCR bind MHC, peptide, or superantigen.

Publications:

Kurucz I, Jost CR, George AJT, Andrew SM D.M. A bacterially-expressed single chain Fv construct from the 2B4 T cell receptor. Proc Nat Acad Sci USA. 1993;90:3830-3834.

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

PROJECT NUMBER

Z01 CB 09297-02 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Characterization of Developmental Mutations Caused by Retroviral Insertion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,  
 PI: Michael Kuehn Expert EIB, NCI

Others: Linda Lowe Biologist EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH  
 Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:  
 0.75

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

To identify and isolate developmental genes, we are carrying out insertional mutagenesis studies in transgenic mice derived from retrovirally infected embryonic stem (ES) cells. We have analyzed a strain segregating 23 proviruses (412 strain) to determine if any are associated with insertional mutations. We have genetic and molecular evidence that 3 of the proviruses cause recessive prenatal lethality. We have circumstantial evidence that at least one additional provirus is associated with an abnormal visible phenotype that is reminiscent of a previously defined mutation, disorganization (Ds). The molecular and phenotypic analysis of these potential insertional mutations is underway.



## Project Description

Major Findings:

The 412 strain segregates 23 proviruses. These were introduced into the germ line by ES cell mediated transgenesis. To determine if any cause insertional mutations in developmentally important genes, we crossed animals heterozygous for a particular provirus and examined the offspring for homozygotes. In 3 cases no homozygotes were found in more than 20 offspring analyzed. We therefore assumed that these 3 proviruses cause recessive prenatal lethal mutations, which potentially identify developmentally interesting genes.

To test this assumption, we are in the process of breeding additional mice that will be used for the visual inspection and histological analysis of mutant embryos at various stages of development. This should be complete by the end of this reporting period.

At the molecular level, we have isolated each of the proviral integration loci as sets of overlapping cosmid clones. We are in the process of searching the 3 different sets of cloned DNA for exons that may represent part or all of each of the mutated genes. We have also begun chromosomal mapping, as a first step in determining possible allelism to known developmental genes. This is being done by identifying regions in the cloned flanking DNA that contain stretches of the simple dinucleotide repeat sequence CA. These regions are subcloned and sequenced. PCR primers are then designed for amplification of the CA repeat region (usually a fragment of approximately 100 bp). Genomic DNA from different mouse strains is then amplified to determine whether the length of the CA region is polymorphic. There are often polymorphisms between strains which have been used to make recombinant inbred (RI) strains. The RI lines can then each be examined to determine the strain distribution pattern of the polymorphism and thus the chromosomal position. In cases where the RI lines are not polymorphic, we examine interspecific backcross mice. These are usually informative because of the high probability for polymorphism between the wild strain and laboratory strain used to generate the panel of backcross mice. Using this approach, the proviral integration site known as 412-k has been mapped to chromosome 8. A mouse was born with a striking anatomical defect: additional digits located on the cranium. Based on this bizarre phenotype, this mouse could represent a recurrence of the mutation, disorganization (Ds). Ds mice show a variety of defects, but the mutation usually has a very low degree of penetrance. We bred this individual to identify any additional abnormal mice, but none were ever found. The mouse was found to be homozygous for 7 of the 412 strain proviruses. Using the procedure described above, we decided to map all 7 to see if any are located on chromosome 14 near the Ds locus. Two of the 7 were found to be X-linked. Of the remaining 5 autosomal insertions, we have cloned 4 and are in the

process of cloning the fifth. So far, we have mapped one of the cloned insertions, 412-a, to chromosome 17 within the T/t complex. The remaining 4 will be mapped by the end of this reporting period. If one turns out to be on chromosome 14 we will carry out higher resolution mapping to more precisely pinpoint the location. In addition we will search for a gene in the flanking DNA as described above.

Publications:

None

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

PROJECT NUMBER

Z01 CB 09298-02 EIB

PERIOD COVERED

October 1, 1993 to September 30, 1993

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

Multiplex Method for Large Scale Insertional Mutagenesis in Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, PI: Michael Kuehn Expert EIB, NCI

Others: Linda Lowe Biologist EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.75

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

C

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

We are implementing an approach to improve the efficiency and applicability of insertional mutagenesis as a method to identify and clone a large number of developmentally important genes. This approach is based on a novel multiplex strategy for analyzing several sets of different proviruses, each present at multiple copy number and all integrated into the germ line of the same transgenic mouse strain. Clones of ES cells have been derived by multiple infection with 6 different retroviral vectors and analyzed to identify ones that average 20 copies of each provirus type (and a total copy number of approximately 120). One clone is now being used to make injection chimeras by a new approach that improves efficiency. We use carrier blastocysts derived from super-ovulated C57/B16 mice. We have shown that higher numbers of embryos can be harvested per donor mouse, as expected. But also, we have shown that these embryos do not differ from ones collected from naturally mated females, in terms of viability after transfer to foster mothers.

## Project Description

Major Findings:

We previously developed an approach to analyze large numbers of proviruses introduced into the germ line by ES cell transgenic techniques. We derived eighteen new retroviral vectors by insertion of different fragments of phage lambda DNA into a basic retroviral vector. We developed protocols to infect ES cells so that individual clones carry from 10 to 20 copies of each of 6 of the vectors. Because each vector has its own unique molecular tag, each small group of 10 to 20 proviruses can be separately analyzed in ES cell clones. This "multiplex" analysis is done by carrying out multiple rounds of probing, stripping and reprobing of the same Southern transfers of DNA, with probes homologous to the specific lambda sequence carried by each vector.

We generated and analyzed a large number of clones. Currently, we are using two of these clones to create ES cell injection chimeras and, hopefully, transgenic mice. We will know by the end of the reporting period whether these clones are germ-line competent. We will screen additional clones for germ-line competence if we are unable to generate germ-line chimeras with these two. Any new transgenic strains we do generate within the reprotng period will be screened for mutations which disrupt development using standard 3 generation backcross breeding programs. Visible recessive mutations will be scored and correlation between a particular retroviral insertion and the mutation will be determined. For recessive prenatal lethal mutations, we will determine which retroviral insertions are not found in the homozygous state in live born progeny of the backcross populstion. We will also cross the transgenics with strains carrying classical mutations, eg. piebald, in an attempt to identify new alleles of these phenotypically identified but as yet uncloned genes.

We are now making injection chimeras by an approach that improves efficiency. To maximize the chance that ES cells will contribute to the germ line of chimeric animals, specific inbred strains of mice are now used as embryo donors. It has been shown that ES cells, derived from the 129/Sv strain, contribute extensively to the germ line and somatic tissues of chimeras made using C57Bl/6 or Balb/c mice as blastocyst donors. However, these inbred strains are not as efficient as previously used outbred strains for blastocyst production. The advantages gained in increased germ-line contribution can be offset by the effort required to harvest sufficient numbers of blastocysts for injection experiments. Superovulation of female mice is widely used in transgenic work for efficient production of fertilized single celled ova. However, this is not the case for production of blastocysts to be used for injection with ES cells. Several investigators have advised against the use of superovulation for blastocyst production, even though early work showed approximately the same viability for blastocysts from spontaneously ovulating mice as from superovulated mice. There have been reports that superovulation results in a greater proportion of abnormal ova and in lower fertility rates and higher loss of embryos during pregnancy compared to spontaneous ovulation.

In light of this conflicting evidence, we examined the question of whether superovulated prepuberal C57Bl/6 mice can be used as embryo donors. We found that this approach not only gives a significant increase in the number of blastocysts recovered per donor female but that there is no difference in viability following embryo transfer to foster mothers. In addition, superovulation allows substantially smaller populations of both male and female mice to be kept on hand. Our results show that superovulation is an efficient method of producing viable blastocysts for ES cell chimera production.

Publications:

Kovacs MS, Lowe L, Kuehn MR. (1993) Use of superovulated mice as embryo donors for ES cell injection chimeras. *Laboratory Animal Science* 199:43; 91-93.

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

PROJECT NUMBER

Z01 CB 09299-01 EIB

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Molecular Characterization of the Nodal Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, PI: Michael Kuehn Expert EIB, NCI  
 Others: Xunlei Zhou Visiting fellow EIB, NCI  
 Lynn Bristol Staff fellow EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH  
 Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:  
 2.5

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

We have characterized a retroviral insertional mutation that causes prenatal lethality. Mutant embryos do not form mesoderm and do not gastrulate. The gene mutated by retroviral insertion has been cloned and identified as a novel member of the TGF-beta superfamily. Further molecular characterization of the gene and gene product is underway.

## Project Description

Major Findings:

The 413-d provirus causes an insertional mutation that results in prenatal lethality. As reported previously, phenotypic analysis of mutant embryos revealed abnormalities beginning at the egg cylinder stage (day 7 of embryogenesis). Mutant embryos at this stage are characterized by hyperplasia of embryonic and extra-embryonic ectoderm. Mutant embryos also do not contain any mesoderm, and show no evidence for a primitive streak. Therefore, the mutation causes all ectodermal cells to continue to proliferate including those that would normally differentiate into mesoderm.

During the reporting period we have cloned the gene mutated by the 413-d provirus. As reported previously, the 413-d provirus as well as flanking cellular sequences were cloned from a cosmid library of genomic DNA. Nine overlapping cosmid clones were isolated using genetic selection for the neo gene contained in the retroviral vector. Together, the clones cover a genomic region totaling more than 60 kb around the site of the 413-d retroviral insertion. To find the gene mutated by proviral insertion, we screened a 7.5 days post coitum (dpc) mouse embryo cDNA library with a cloned genomic DNA fragment flanking the provirus. This fragment contains recognition sites for NotI, BssH II and Sac II which are characteristic of CpG islands, often found lying close to genes. From a screen of  $1 \times 10^6$  recombinant phages we isolated a single 1800 base pair (bp) long cDNA clone which maps to both sides of the 413-d provirus. The cDNA contains a long open reading frame (ORF) starting at the 5' end. Although the ORF does not begin with a MET codon, sequence analysis of cloned genomic DNA showed that the ORF continues to an in-frame MET codon 24 bp upstream. Examination of the deduced protein sequence revealed extensive homology to the DVR (decapentaplegic-Vg-1-Bone Morphogenetic Protein(BMP)-related) and activin/inhibin subgroups of the TGF-beta superfamily. These secreted proteins act as signaling molecules mediating cellular interactions in many tissues during development. TGF-beta-like proteins are most highly conserved at their carboxy termini. This region, encompassing the mature form of the protein, is generated by proteolytic cleavage of a larger precursor. An alignment of the predicted carboxy terminal 110 amino acids of this gene showed it to be from 34% to 39% identical in the conserved region to other superfamily members, and about 25% identical to TGF-beta itself. The closest evolutionary relationships are to the gene for the inhibin betaB chain and to BMP-3.

To determine the in vivo expression pattern of this new gene, both reverse-transcriptase PCR (RT-PCR) and whole mount in situ hybridization were carried out on wild type embryos around the time of gastrulation. In the mouse, gastrulation starts at approximately 6.5 dpc with the formation of the primitive streak at the junction of the embryonic and extraembryonic ectoderm at the posterior of the embryo. As development proceeds the primitive streak elongates due to growth of the egg cylinder and movement of cells from the embryonic ectoderm into the posterior of the streak.

As a result, the anterior of the streak comes to lie at the distal tip of the egg cylinder and at this location a discrete structure known as the node becomes visible at approximately 7.5 dpc. Cells leaving the node give rise to definitive endoderm, notochord and paraxial mesoderm. Using RT-PCR, nodal RNA is detectable in total RNA prepared from pre-streak and very early streak embryos, although the amount of amplified product is far below that found for later stage embryos. The first detectable expression of nodal by whole mount *in situ* hybridization coincides with the appearance of the node. The hybridization is highly localized and can be seen as a ring of staining around the node. This pattern persists through the neural plate/head fold stage, but no signal is detected by 8.5 dpc, coinciding with the disappearance of the node as a distinct structure. It is because of this localized expression in the node that we have proposed the name nodal for this gene.

To further characterize the nodal gene and gene product, we have generated anti-peptide antibodies against specific regions of the deduced protein sequence. These are currently being characterized. In addition, we are attempting to express the nodal gene in CHO cells, so that we can eventually purify sufficient amounts of protein to attempt to expression clone the nodal receptor. A composite genomic/cDNA fragment was generated by PCR and cloned into the pcDNA expression vector. This was transfected into CHO cells and clones were selected. Characterization of the clone using the antibodies should be complete by the end of the reporting period.

Publications:

Iannaccone PM, Zhou X, Khokha M, Boucher D, Kuehn MR. Insertional mutation of a gene involved in growth regulation of the early mouse embryo. *Developmental Dynamics* 1992;194:198-208.

Zhou X, Sasaki H, Lowe L, Hogan BLM, Kuehn MR. Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. *Nature* 1993;361:543-547.



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

PROJECT NUMBER

Z01 Cb 09400-01 EIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Role of MHC class I in the generation of autoimmune diseases

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

P.I.:	Dinah Singer	Section Chief	EIB, NCI
Others:	Leonard Kohn	Senior Investigator	NIDDK
	Edna Mozes	Guest Researcher	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

Molecular Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

MHC class I genes are subject to both homeostatic, tissue-specific regulation and dynamic regulation. Among the mechanisms of dynamic regulation are those mediated by hormones that either increase or decrease transcription of the genes. The observation that TSH leads to decreased class I transcription, whereas thyroid hormone leads to increased transcription, led to the hypothesis that failure to appropriately regulate MHC class I molecules may play a pivotal role in the generation of autoimmune disease. Consistent with this hypothesis, it was demonstrated that in an experimental model of autoimmunity, animals that fail to express class I molecules are resistant to disease.

## Project Description

Major Findings:

Experimental systemic lupus erthematosus (SLE) can be induced in mice by immunization with a human monoclonal anti-DNA antibody that bears a common idiotype (16/6Id). These mice generate antibodies to 16/6Id, antibodies to DNA, and antibodies to nuclear antigens. Subsequently, manifestations of SLE develop: leukopenia, proteinuria and immune complex deposits in the kidney. In contrast, following immunization with 16/6Id, class I<sup>-</sup> mice generated anti-16/6 Id antibodies, but did not generate anti-DNA or anti-nuclear antigen antibodies. Furthermore, they did not develop any of the clinical manifestations. These results indicate a major role for MHC class I molecules in the induction of SLE.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09401-01 EIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

HIV-mediated repression of MHC class I gene expression

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

P.I.:	Dinah Singer	Section Chief	EIB, NCI
Others:	T.K. Howcroft	Staff Fellow	EIB, NCI
	Julie Brown	Staff Fellow	EIB, NCI
	David Nikodem	Tech	EIB, NCI
	Malcolm Martin	Senior Investigator	LMM, NIAID
	Klaus Strebel	Senior Staff Fellow	LMM, NIAID

## COOPERATING UNITS (if any)

## LAB/BRANCH

Experimental Immunology Branch

## SECTION

Molecular Regulation Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## 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.)

MHC class I molecules are the major receptors for viral peptides and serve as targets for specific cytotoxic T lymphocytes. HIV-1 specifically decreased activity of an MHC class I gene promoter up to 12-fold. Repression was effected by the HIV-1 Tat protein derived from a spliced viral transcript (two-exon Tat). These studies define an activity for two-exon Tat distinct from that of one-exon Tat and suggest a mechanism whereby HIV-1-infected cells might be able to avoid immune surveillance, allowing the virus to persist in the infected host.

## Project Description

Major Findings:

Many viruses, particularly tumorigenic viruses, actively decrease levels of cell surface MHC class I expression. It has been proposed that this is a mechanism to evade host immune surveillance. Therefore, the effect of HIV gene products on MHC class I expression was examined. Transient transfection of human HeLa cells with a proviral construct containing all HIV genes except for gag and pol resulted in a significant decrease in class I expression in transfected, but not non-transfected cells. Most, if not all, of this reduction can be accounted for by decreased transcription from the class I promoter, as determined by co-transfection assays of the HIV proviral construct with a class I promoter reporter construct. The HIV responsive element within the class I promoter was mapped to within 68 bp of transcription initiation, to a segment containing only the canonical promoter elements.

Using a series of deletion mutants of the HIV proviral construct that individually eliminated vif, vpu, nef, rev, vpr, and env, it was found that none of these products is responsible for the transcriptional repression. Furthermore, the one-exon form of Tat, which is expressed after the onset of Rev synthesis, also was incapable of down-regulating class I transcription. However, the two-exon form of Tat, p16, which is derived from the fully spliced viral genome, is fully capable of decreasing class I transcription. This effect on initiation occurs in the absence of the viral TAR sequence and is promoter specific. It also establishes a role for the second-exon of Tat. These findings lead to the speculation that class I gene expression is reduced during a persistent phase of HIV infection, providing a viral reservoir which is hidden from the immune system.

Publications:

Howcroft TK, Strebel K, Martin M, Singer DS. Repression of MHC Class I Gene Promoter Activity by Two-Exon Tat of HIV. Science 1993;260:1320-1323.

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

PROJECT NUMBER

Z01 CB 09402-01 EIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 T Cell Immune Dysregulation in Cancer

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

PI:	G. M. Shearer	Section Chief	EIB, NCI
Others:	M. Clerici	Visiting Scientist	EIB, NCI
	R. E. Gress	Section Chief	EIB, NCI
	D. Longo	Head	BRMP, NCI

COOPERATING UNITS (if any)

M. L. Villa, Università di Milano, Milano, Italy

LAB/BRANCH

Experimental Immunology Branch

SECTION

Cell Mediated Immunity Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human                       (b) Human tissues     (c) Neither                      B, A, D  
 (a1) Minors  
 (a2) Interviews

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

T helper cell (Th) function was assessed using peripheral blood leukocyte (PBL) from 47 patients newly diagnosed with Hodgkins disease (HD). The PBL were stimulated in vitro with recall antigens (REC), HLA alloantigens (ALLO) and phytohemagglutinin (PHA). Four distinct patterns of Th responsiveness were detected: patients who responded to all three stimuli; patients who did not respond to REC; patients who responded only to PHA; and patients whose PBL were refractory to all three stimuli. The more severely immune-compromised patients exhibited more severe hematologic parameters of HD.

## Project Description

Major Findings:

This laboratory has found a correlation between Th immune dysfunction and disease progression in HIV-infected individuals, and has also demonstrated that certain pathways of T cell activation are correlated with human solid organ allograft rejection. We have now begun to investigate the possibility that certain cancers are also accompanied by Th immune dysregulation.

recently diagnosed patients with Hodgkin's disease (HD) were tested for Th function by in vitro stimulation of their PBL with REC, ALLO, and PHA. Of the initial 47 patients tested: 40% responded to all three stimuli; 26% responded to ALLO and PHA, but not to REC; 19% responded to PHA but not to REC; and 15% failed to respond to all three stimuli. None of the 34 healthy control donors tested exhibited any of the above defects. Patients with more severely impaired Th immunity tended to present with more clinically advanced stages of HD, for example, clinical stages III and IV.

The above preliminary finding raises the possibility that our sensitive Th assay can be used to supplement diagnosis of certain types of cancer. Studies are in progress to determine whether other types of cancer will be associated with similar T cell immune dysregulation.

Publications:

None

## Annual Report

### LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY FY 1993

#### EXPERIMENTAL ONCOLOGY SECTION (J. Schlom, Chief).

The EOS is involved in two major areas of research: (a) the generation, characterization, genetic modification and use of monoclonal antibodies (MAbs) directed against human carcinoma associated antigens, and (b) the design, construction, analysis and potential use of recombinant vaccines for the active specific immunotherapy of human carcinomas. There are several areas of overlap between these two research programs.

There are five Working Groups within the EOS. Although there is some overlap in the studies being carried out as a result of research collaborations, studies can best be divided into five major areas: Studies of the Monoclonal Antibody Working Group involve the generation and characterization of MAbs to human carcinoma associated antigens and the characterization of the reactive antigens. MAbs are being evaluated which react to three carcinoma associated antigens: (i) TAG-72 (Tumor Associated Glycoprotein) recognized by MAbs B72.3 and CC49 among others, (ii) carcinoembryonic antigen (CEA) recognized by the COL MAbs, and (iii) the colon carcinoma associated antigen recognized by MAb D612. This project also involves preclinical studies which are designed to define those parameters and phenomena which will lead to the effective use of these MAbs in various modalities in the diagnosis and therapy of a range of human carcinomas. To date, most emphasis has been placed on the use of radiolabeled MAbs; studies are currently underway in the analysis of MAb forms which mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and with drug-conjugated MAbs. Numerous collaborative diagnostic and therapeutic clinical trials are currently ongoing to evaluate the radioimmunoconjugates developed to date.

Studies of the Recombinant Immunoglobulin Working Group involve the design, construction, and analysis of novel recombinant immunoglobulin molecules toward the study of structure-function relationships of immunoglobulins and the design of molecules that will be more effective in the diagnosis and therapy of human carcinomas. It has become clear from a range of clinical trials that such properties as immunogenicity, plasma clearance, metabolic patterns, and tumor penetrance of intact murine immunoglobulins must be improved for more efficient clinical use. This project has involved the design and modification of numerous recombinant immunoglobulin forms toward this end. Also being studied is the potential use of immunoglobulin genes in "gene therapy" and the construction of molecules that may be useful in our vaccine program.

One of the major potential problems in the immunotherapy of cancer is the antigenic heterogeneity of many tumor masses. A major goal of the Cytokine Working Group has been the design and evaluation of various biologic response modifiers that can minimize or eliminate this problem. This phenomenon, moreover, has implications both in the MAb program and the vaccine program. To date, several cytokines and other molecules have been identified which can selectively enhance tumor associated antigen expression on tumor cells and, as has been shown both *in vitro* and *in vivo* in experimental models, leads to better MAb tumor targeting and more effective therapeutic responses. Several collaborative clinical studies are in progress to further evaluate this phenomenon. Cytokine studies are also in progress in an attempt to reduce dose limiting hematological toxicities.

Studies of the Recombinant Vaccine Working Group involve the generation and characterization of novel recombinant vaccines toward the active specific immunotherapy of human carcinomas. To date, most emphasis has been placed on the construction of recombinant vaccinia viruses containing human tumor associated genes and in the development of recombinant forms of these gene products in baculovirus. Our initial studies with a recombinant vaccinia virus containing the human CEA gene has proved to be immunogenic and

safe in both rodents and primates, and to elicit good anti-tumor responses in a rodent model. Studies of the Immunology Working Group involve a detailed analysis of host immune responses to the recombinant vaccines in mice, non-human primates and in patients in up-coming clinical trials. Among the tumor associated targets to be evaluated via recombinant vaccines are CEA, human prostate specific antigen, and the point-mutated human *ras* oncogenes.

**MONOCLONAL ANTIBODY WORKING GROUP : Development, Characterization and Utilization of Monoclonal Antibodies to Carcinoma Associated Antigens. (J. Schlom)**

Progress has been made in the characterization of MABs to three carcinoma associated antigens and the potential use of these MABs in both the diagnosis and therapy of a range of carcinomas. The three antigens are (a) TAG-72, a high molecular weight mucin expressed in gastrointestinal, breast, ovarian, endometrial, prostate and non-small cell lung cancers, which is recognized by MABs B72.3, CC49 and CC83; (b) carcinoembryonic antigen (CEA) a 180,000 D glycoprotein, expressed in gastrointestinal, breast and non-small cell lung cancer, which is recognized by MABs COL-1 through 15 and (c) a 48,000 D glycoprotein expressed on colon carcinomas and normal colon, which is recognized by MAB D612. To test the hypothesis of whether high affinity MABs have a greater therapeutic efficacy, the anti-tumor activity of three radiolabeled anti-TAG-72 MABs in a human colon cancer xenograft model was analyzed. At all doses examined, the higher affinity MABs (CC49 and CC83) demonstrated greater anti-tumor effects than B72.3. A novel radioimmunoconjugate, lutetium (<sup>177</sup>Lu)-labeled CC49 was analyzed in this same experimental model. <sup>177</sup>Lu is a rare earth lanthanide with unique radiopharmaceutical properties. Strong anti-tumor effects were observed with minimal toxicity. A single chain sFv has been constructed from MAB CC49 and has shown good tumor targeting properties, and rapid plasma and whole body clearance in a xenograft model. Quantitative autoradiographic analyses revealed that penetration through tumors with the sFv was extremely rapid and more homogeneous as compared to intact IgG. It has also been determined that a <sup>177</sup>Lu-labeled sFv had a much different metabolic profile than iodinated sFv CC49. These studies have implications in the design of potential clinical trials. Employing the CA72-4 immunoassay to detect the TAG-72 antigen in the serum of cancer patients, it was shown that combined analyses of TAG-72 and the 19-9 antigen levels significantly increased the identification of patients with gastric cancer, while combined use of assays to detect TAG-72 and CEA increased positive rates for colorectal cancer patients, in both cases with no substantial increase in false positives. Using a series of cell lines transduced with the CEA and related genes as controls, it was shown that in addition to GI carcinomas, CEA is expressed in approximately 50% of human breast cancers and 70% of non-small cell lung cancers. Further evaluation of the anti-CEA MAB COL-1 revealed that <sup>131</sup>I-COL-1 could efficiently target and eliminate the growth of established human tumors in a xenograft model. The antigen recognized by MAB D612 was further characterized. The ability of MABs D612 and chimeric B72.3 and CC49 bearing the human  $\gamma$ 1 backbone to mediate antibody-directed cell-mediated cytotoxicity was defined, along with the ability of selected cytokines to enhance these activities. Numerous collaborative Phase I and II clinical trials with MABs B72.3, CC49, COL-1 and D612 have been completed or are in progress.

**RECOMBINANT IMMUNOGLOBULIN WORKING GROUP: Novel Recombinant Immunoglobulin Molecules for Cancer Therapy and Diagnosis. (S. Kashmiri and P. Horan Hand)**

The major goal of this research effort is to design, construct and generate novel recombinant immunological molecules for the diagnosis and therapy of human cancers. In ongoing clinical trials, the anti-TAG-72 MABs, B72.3 and CC49, MAB D612 and an anti-CEA MAB, COL-1, have shown various degrees of potential for being developed into diagnostic and therapeutic reagents. However, the usefulness of murine MABs for in vivo diagnosis and therapy is limited because of their immunogenicity. To reduce this potential problem we have



developed mouse-human chimeric (c) MAbs, including cB72.3 ( $\gamma$ 1), using recombinant DNA techniques. In an effort to optimize the pharmacokinetics of plasma clearance and to maximize the efficiency of localization of, and penetration into, tumors novel chimeric immunoglobulin variants have been developed; these include aglycosylated cB72.3 ( $\gamma$ 1) MAb and constant region domain-deleted variants of cB72.3 ( $\gamma$ 1). In comparison with the cB72.3 MAb, the CH2 domain-deleted cMAb demonstrated a faster clearance rate and a more rapid tumor targeting. For second generation anti-TAG-72 MAbs, the heavy and light chain genes of MAb CC49 and CC83 have been cloned, sequenced and inserted into retroviral vectors. Recently, the cCC49 ( $\gamma$ 1) has been expressed and purified. We have also developed a single gene encoded Ig molecule of cCC49 which has retained effector functions. We have also developed a cD612 MAb which has been expressed and secreted by a human T cell line. The secreted immunoglobulin retained its antigen-binding properties and its ability to mediate antibody-dependent cell-mediated cytotoxicity against human tumor cells. To our knowledge, this is the first demonstration of the production of an IgG by human T cells and opens the possibility of a therapeutic approach in which T-cells secrete humanized anti-tumor MAb capable of mediating antibody-dependent cell-mediated cytotoxicity at the tumor site.

#### **CYTOKINE WORKING GROUP: Cytokine-based Modalities to Enhance Antibody and Cell-Mediated Tumor Cell Killing. (J. Greiner)**

Tumor antigen heterogeneity may at times impact on MAb targeting, thereby compromising its overall effect as an immunodiagnostic and/or immunotherapeutic agent. This area of research involves the study of cytokines which selectively up-regulate human tumor antigen expression. We have reported that the recombinant interferons can selectively enhance the expression of carcinoembryonic antigen (CEA), tumor-associated glycoprotein (TAG)-72 and a potentially novel Mr 110,000 antigen, in a variety of human carcinomas. Interferon- $\gamma$ , in particular, substantially increases TAG-72 and CEA expression in human tumor xenografts leading to an increased tumor MAb localization. That ability of interferon-gamma to enhance MAb targeting to the tumor site provided a significant therapeutic advantage over MAb administration alone. Those preclinical findings provided the rationale to collaboratively investigate the ability of interferon to augment human tumor antigen expression in a clinical setting. Patients diagnosed with either ovarian or gastrointestinal carcinoma with secondary ascites were given weekly i.p. doses of interferon- $\gamma$  (0.1 to 100 MU). Analyses of TAG-72 and CEA expression on isolated malignant ascites cells showed a dramatic increase in the expression of both tumor antigens as a result of interferon- $\gamma$ . In some cases, interferon- $\gamma$  increased the percentage of tumor cells expressing either TAG-72 or CEA from 10% to >90% and those increases were observed at low interferon- $\gamma$  doses (i.e., 0.1 to 1.0 MU), which were well tolerated by all patients. Thus, both experimental and early clinical results provide substantial support for the use of a cytokine, such as an interferon, as an adjuvant to enhance MAb binding to human carcinoma cell populations.

#### **RECOMBINANT VACCINE WORKING GROUP: Recombinant Vaccines for Active Specific Immunotherapy of Human Carcinoma. (J. Kantor)**

Certain tumor associated antigens (TAAs) represent potential targets for active specific immunotherapy. Human carcinoembryonic antigen (CEA) is a 180 kd glycoprotein which is overexpressed in human colorectal, gastric, pancreatic, breast and non-small cell lung carcinomas. CEA is an oncofetal protein and is considered to be weakly immunogenic in humans. Humoral or cell mediated responses to CEA have not been well documented in normal or cancer patients. The copresentation of CEA with a strong immunogen such as vaccinia virus would represent a logical approach to inducing anti CEA responses for tumor immunotherapy. We have constructed and characterized a recombinant vaccinia virus expressing human CEA and have used it as an immunogen to study its effect on tumor growth in mice bearing CEA expressing tumors. Rodent tumors do not express CEA. In order to develop a model system for active anti-CEA therapies, we have transduced a mouse colon adenocarcinoma cell line, MC-38, with human

CEA. These tumors grow in syngeneic C57BL/6 mice and will eventually kill the animal. We have used this tumor model to evaluate the efficacy of the CEA recombinant vaccine to prevent tumor growth in mice and its ability to elicit cell mediated and humoral anti-CEA immune responses. Animals immunized with the recombinant vaccine were resistant to challenge with syngeneic tumor cells expressing CEA. Moreover, when mice having a palpable tumor burden were immunized with the recombinant vaccine, tumor growth was greatly reduced or eliminated. The recombinant CEA vaccine immunized animals developed anti-CEA antibody titers and demonstrated a specific cellular response to CEA and CEA expressing tumor cells. No toxicity was observed in these animals. Immunogenicity and safety of this recombinant vaccine was tested in non-human primates. Animals immunized with the recombinant vaccine developed strong anti-CEA antibody responses and specific DTH responses. PBLs from immunized monkeys were found to proliferate in response to CEA stimulation. Blood counts and differentials and hepatic and renal chemistries remained normal in all animals throughout the study and for up to 1 year following the primary immunization.

**IMMUNOLOGY WORKING GROUP: Host Immune Responses to Human Carcinoma Antigens Induced by Recombinant Vaccines.** (S. Abrams and K. Tsang)

This project involves a detailed analysis of host immune responses to a spectrum of recombinant vaccines derived from viral vectors such as vaccinia, and from recombinant proteins, peptides, and/or anti-idiotypic immunoglobulins. The over-expression of tumor-associated antigen and/or the neo-expression of tumor-specific epitopes may represent selective or unique targets for immune recognition, particularly by T lymphocytes which have been implicated as integral effector elements for host anti-tumor activity. Thus, we have begun to evaluate the repertoire of host cellular immune responses induced by active immunization to CEA and point-mutated *ras* p21. In a murine C57BL/6(H-2<sup>b</sup>) model, we have examined whether active immunization with a recombinant vaccinia virus expressing human CEA (rV-CEA) could induce T cell responses, which might correlate with and/or participate in the tumor rejection mechanism. Overall, we have shown that (1) T cells from rV-CEA mice elicited antigen specific proliferation to soluble CEA protein; (2) T cells from rV-CEA mice mediated specific lysis of CEA<sup>+</sup>-bearing tumor target cells; and (3) splenocytes from rV-CEA mice expressed anti-tumor activity through adoptive immunotherapy. Similarly, we have shown that lymphocytes from rV-CEA immunized rhesus monkeys displayed antigen-specific proliferation responses. More recently, we have examined whether active immunization with short synthetic peptides, which mimic point-mutated epitopes of *ras* p21, could induce T cell responses in a murine BALB/c model. Overall, we have shown that (1) mice immunized with a *ras* 13-mer peptide, containing the substitution of glycine at position 12 for valine, demonstrated specific T cell proliferation to the immunization peptide. No autoimmune response was detectable to the normal *ras* p21 (glycine) sequence; (2) CD4<sup>+</sup>T cells (lines and clones) were established *in vitro*, which retained peptide specificity; and (3) CD4<sup>+</sup> T cells secreted a spectrum of cytokines (e.g., IL2, IFN- $\gamma$ , TNF or GM-CSF) and some effectors expressed cytotoxicity against tumor target cells incubated with the specific peptide. Taken collectively, we demonstrate that a rV-CEA construct and point-mutated *ras* p21 peptides elicit specific cellular immune responses in animal models.

We have also investigated whether human T lymphocytes are able to distinguish the determinants of the point-mutated *ras* p21 proteins from normal *ras* p21. Cellular immunity to three synthetic peptides representing amino acids 5-17 of mutated *ras* p21 proteins with an exchange of normal glycine (G12) at position 12 by valine (V12), cysteine (C12) or aspartic acid (D12) was studied. T cell lines specific for peptides V12, C12, and D12 (but not G12) were able to be established from normal peripheral blood lymphocytes. The specificity of the T cell lines were assayed by T cell proliferation and production of cytokines. Cytotoxicity was demonstrated using as targets autologous EBV transformed B cells, pulsed with specific mutated *ras* peptides, and autologous EBV transformed B cells transfected with a vector carrying p21 *ras* protein constructs. T cells were MHC class II restricted. The results demonstrated that a

human T cell specific immune response to point-mutated *ras* p21 proteins bearing a single amino acid substitution can be elicited from PBL, suggesting a potential for specific immunotherapy of human cancers.

#### ONCOGENE WORKING GROUP (Dr. M. L. Cutler)

##### Expression Cloning of Genes Capable of Suppressing *ras* Transformation.

The major focus of this project is determining the role of negative regulation of signal transduction pathways in cellular growth and transformation. Specifically, we have sought to identify genes which can negatively regulate signal transduction induced by an activated *ras* gene. We have utilized an expression cloning strategy to isolate cDNA molecules which can suppress *ras* transformation. We are characterizing the mechanisms and pathways necessary for their phenotypic expression. To identify such genes, a cDNA library was constructed in a eukaryotic expression vector using RNA from a *ras* revertant cell line and transfected into the *ras* transformed cell line, DT. Following selection for cells which had taken up cDNA, phenotypically "flat" primary transfectants were isolated. The cDNAs recovered from these transfectants were assayed by a secondary round of screening for *ras* suppresser activity on DT cells. With this procedure more than 100 primary transfectants have been isolated and expanded into cell lines. cDNA have been recovered from more than 20 of these cell lines and tested in a secondary screening assay. Two cDNAs isolated from primary transfectants have been found on secondary screening to be capable of suppressing the *ras* transformed phenotype.

The first of these cDNA encodes a small RNA, 4.5S RNA. High levels of 4.5S RNA are found in *ras* revertant cell lines and reduced levels in *ras* transformed cell lines compared to the level of this RNA in normal rodent fibroblasts. High levels of this RNA in the *ras* revertants appears to be a result of increased transcription and increased stability of the 4.5S RNA; whereas the low level in *ras* transformed cells is attributable to change in the rate of transcription. The level of 4.5S RNA is also reduced in fibroblasts transformed by *src* and *mos* oncogenes. While the mechanism by which high level expression of 4.5S RNA suppresses the transformed phenotype is not known, there are several possible roles for this molecule that are of immediate interest.

In addition, another recovered cDNA, referred to as *rsp-1*, is a novel gene which specifically suppresses v-Ki-*ras* and v-Ha-*ras* transformation of fibroblasts and epithelial cells. The *rsp-1* protein contains a series of leucine based repeats homologous to those found in the putative *ras* binding region of *Sa. cerevisiae* adenyl cyclase. These findings suggest that *rsp-1* may physically associate with *ras* p21 or other *ras* associated proteins and hence disrupt *ras* signal transduction. Alternatively, *rsp-1* itself may be a *ras* target and high level expression of *rsp-1* in our assay may disrupt normal signaling. *Rsp-1* is a phylogenetically highly conserved molecule; cloning and sequencing of the human *rsp-1* cDNA revealed that the human *rsp-1* protein is 96% homologous to the mouse *rsp-1*. Screening of over 100 cell lines and tissue, both human and rodent, revealed that *rsp-1* RNA expression is ubiquitous. The human *rsp-1* gene has been localized to human chromosome 10, and we have identified a human tumor cell line, and primary human tumors, with alterations in *rsp-1* protein expression. Our current efforts are aimed at determining the mechanisms by which this molecule can disrupt *ras* signal transduction in our assay and determining if it can serve as a loss of function mutation or a mutation activating the *ras* signal transduction pathway.

Secondary screening of cDNA molecules recovered from our primary transfectants indicates that at least 10% of these molecules have the ability to suppress *ras* transformation when expressed at a high level under the control of a heterologous promoter. In addition to 4.5S RNA and *rsp-1* we have isolated a cDNA with homology to the known *ras* suppresser K-*rev-1*.

#### ENDOCRINOLOGY WORKING GROUP (Dr. B. Vonderhaar)

##### Hormones, Antihormones and Growth Factors in Mammary Development and Tumorigenesis.

Our interest has been on the interactions of hormones, antihormones, and growth factors in mammary gland growth and development and tumorigenesis. We used a combination of *in vivo* and *in vitro* approaches to establish the physiological relevance of our findings. We have used whole organ cultures to explore normal glandular development. We determined that growth hormone can partially substitute for prolactin (Prl) in both morphogenesis and differentiation. Priming of the mice with estrogen/progesterone or progesterone alone increases DNA synthesis by decreasing the growth inhibitory effects of TGF- $\beta$ 1 in the uncultured gland. We showed that when primary cultures of mammary cells, infected with a retrovirus carrying an inducible gene for TGF- $\beta$ 1 were transplanted into cleared mammary fat pads of syngeneic mice, only bulbous end buds with no or very truncated ducts resulted.

The same hormones and growth factors involved in the development of the normal mammary gland are important in mammary cancer. We originally showed that the Prl receptor exists in at least 2 forms, a 90 kDa and a 40 kDa protein. We now have demonstrated that monoclonal antibody B6.2, developed in LTIB, recognizes the Prl receptor(s) on human breast cancer cell lines. The binding site for both Prl and B6.2 contains an essential N-linked carbohydrate.

We have shown that the nonsteroidal antiestrogens such as tamoxifen (TAM) and nafoxidine, acting through the antilactogen binding site (ALBS) inhibit the binding of Prl to normal mouse mammary membranes. In addition to inhibition of Prl binding, TAM also prevents the Prl-induced accumulation of caseins by cultured mouse mammary explants. By Lineweaver-Burk analysis we found that TAM is a competitive inhibitor of Prl binding. Binding of TAM to the ALBS is effectively inhibited by anti-Prl receptor antibody. Using TAM-Sepharose and Prl-Sepharose affinity resins, we found that the 90 kDa Prl receptor and the ALBS co-purify. Mouse 3T3 cells stably transfected with the gene for the 90 kDa Prl receptor simultaneously acquire the ability to bind Prl and TAM. Taken together these data suggest that the ALBS may be one form of the Prl receptor and that TAM and the lactogenic hormones may share a common binding site.

#### **TUMOR GROWTH FACTORS SECTION (Dr. D. Salomon, Chief)** **EGF Related Peptides in Breast and Colon Cancer.**

This laboratory is engaged in studying the biology of epidermal growth factor (EGF)-related peptides such as transforming growth factor  $\alpha$  (TGF $\alpha$ ) in the pathogenesis of breast and colorectal cancer. Over the last four years our laboratory has demonstrated that a number of different activated cellular protooncogenes or viral oncogenes and specific mammotrophic hormones such as estrogen can up regulate the expression of TGF $\alpha$  which can then function as an autocrine growth factor. Specifically, we have shown that MCF-10A human mammary epithelial cells are mitogenically responsive to exogenous EGF or TGF $\alpha$  and that transformation of these cells with a point-mutated c-Ha-ras protooncogene but not with a c-erb B-2 protooncogene results in a loss in mitogenic responsiveness to exogenous EGF and in an increase in the expression of endogenous TGF $\alpha$ . In this context, overexpression of a human TGF $\alpha$  cDNA in these cells and in NOG-8 or HC-11 mouse mammary epithelial cells can lead to their *in vitro* and in certain cases *in vivo* transformation. Addition of an anti-EGF receptor blocking antibody or an anti-TGF $\alpha$  neutralizing antibody can partially or completely inhibit the growth of the Ha-ras or TGF $\alpha$  transformed cells. Physiological, growth-promoting concentrations of 17 $\beta$ -estradiol (E2) can increase the expression of TGF $\alpha$  mRNA and protein in estrogen-responsive human breast cancer cell lines such as MCF-7 or ZR-75-1 cells. Transient transfection assays in MCF-7 or ZR-75-1 cells using a plasmid containing the TGF $\alpha$  promoter ligated to either the chloramphenicol acetyltransferase or luciferase genes have demonstrated that physiological concentrations of E2 can induce a 5- to 50-fold increase in the activity of these reporter genes suggesting that the TGF $\alpha$  promoter contains a *cis*-acting estrogen-responsive element(s). MCF-7 or ZR-75-1 cells have also been infected with a recombinant amphotropic TGF $\alpha$  antisense mRNA expression vector. Expression of this antisense mRNA lead to a reduction in estrogen-induced TGF $\alpha$  protein production and to an equivalent degree of inhibition of E2-

induced proliferation in these cells. More recently, we have been studying the biology of other EGF-related peptides such as amphiregulin (AR), cripto-1 (CR-1) and heregulin a (HRG $\alpha$ ). Specific mRNA and immunoreactivity for AR and CR-1 have been detected in approximately 50 to 80% of primary and metastatic human colorectal tumors, whereas only 5% of normal adjacent colon or liver tissue express these genes. Likewise, immunoreactive AR and CR-1 was detected in approximately 70% of primary human breast tumors at a level that exceeded the level found in adjacent normal mammary epithelium.

**CELLULAR BIOCHEMISTRY SECTION (Dr. Y. S. Cho-Chung, Chief)**  
**The Role of Cyclic AMP in the Control of Cell Proliferation and Differentiation.**

It was discovered that site-selective cAMP analogs can act as novel biological agents capable of inducing growth inhibition and differentiation in a broad spectrum of human cancer cell lines, including carcinomas, sarcomas and leukemias, without causing cytotoxicity. 8-Cl-cAMP, the most potent site-selective cAMP analog, for the first time in cAMP research was selected as a preclinical Phase I antineoplastic agent of the National Cancer Institute (January, 1988). The discovery rendered a critical assessment that the potency of a cAMP analog in growth inhibition depends on the analog's ability to selectively modulate the RI and RII regulatory subunits of cAMP-dependent protein kinase--precisely, down-regulation of RI with up-regulation of RII leading to the restoration of the normal balance of these cAMP-transducing proteins in cancer cells.

The use of antisense strategy and retroviral vector-mediated gene transfer technology provided direct evidence that the RI and RII regulatory subunits have opposite roles in cell growth and differentiation; RI being growth stimulatory while RII is a growth-inhibitory and differentiation-inducing protein. As RI expression is enhanced during chemical or viral carcinogenesis, in human cancer cell lines, in primary human tumors, and in multidrug-resistant (MDR) cancer cells as opposed to non-MDR parental cells, it is a target for cancer diagnosis and therapy.

The cellular events underlying growth inhibition and differentiation of cancer cells induced by 8-Cl-cAMP include a rapid nuclear translocation of RII $\beta$ , and such translocation of RII $\beta$  into the nucleus correlates with an increase in transcription factors in cancer cells that bind specifically to cAMP response element (CRE). Thus, the mechanism of action of 8-Cl-cAMP in the suppression of malignancy may involve the restoration of normal gene transcription in cancer cells where the RII $\beta$  cAMP receptor plays an important role.

By the use of site-directed mutagenesis technology, the structure-function analysis of RI and RII is currently underway. The RI and RII are distinguished by their autophosphorylation and nuclear translocation properties. RII has an autophosphorylation site at a proteolytically sensitive hinge region around the R and C interaction site while RI has a pseudo-phosphorylation site. The RII but not the RI contains a nuclear location signal, K K R K. The autophosphorylation and nuclear location sequences are either point-mutated in RII $\beta$  or introduced into RI $\alpha$  to specifically assess the role of these sequences in the growth regulatory function.

**ONCOGENETICS SECTION (Dr. R. Callahan, Chief)**  
**Identification and Characterization of Mutations in Breast Cancer.**

The Oncogenetics Section has focused its efforts on the identification and characterization of frequently occurring mutations in mouse mammary tumor virus (MMTV)-induced mouse mammary tumors as well as in primary human breast tumors. Activation of the *Int-3* locus was first detected by our laboratory in the CZECHII mouse mammary tumors. Nucleotide sequence analysis of the activated *Int-3* cDNA demonstrated that it is most similar to the *Notch* gene of *Drosophila melanogaster*. Transgenic mice harboring a genomic tumor DNA fragment consisting of the MMTV LTR and the flanking *Int-3* cellular sequences develop focal and often multiple poorly differentiated mammary and salivary adenocarcinomas. In other work we have identified a new common integration (designated *Int-6*) site for MMTV in the CZZ-1 mammary

hyperplastic outgrowth line. The *Int-6* locus is located on chromosome 15 and encodes a 1.4 kb RNA species. During the past four years we have expanded our study of primary human breast tumors and have found 9 regions of the cellular genome that are frequently affected by loss of heterozygosity (LOH), including chromosomes 1p, 1q, 3p, 7q, 11p, 13q, 17p, 17q, and 18q. In most cases LOH at a particular locus was associated with tumors bearing more aggressive characteristics. We have begun to look for the target genes affected by LOH; one of these is the *TP53* gene which is located on chromosome 17p13. We have examined a panel of 121 primary breast tumors which had also been typed for their proliferative index. The overall frequency of *TP53* mutations was 29% and their location within the gene was evenly distributed between exons 5 through exon 8. Stratification of the data based on the exon in which the mutation occurred showed that there is an association between proliferative index and tumors having a mutation in either exon 5 ( $p=0.0002$ ) or exon 6 ( $p=0.05$ ), but not exons 7 or 8.

**CELLULAR AND MOLECULAR PHYSIOLOGY SECTION (Dr. H. COOPER): Cytoskeletal Proteins in Oncogenes Transformation and Human Neoplasia** We have continued our studies on the relationship of suppression of tropomyosin (TM) synthesis to neoplastic transformation. Previous observations have led us to hypothesize that TM suppression is a causal event in neoplastic transformation. We have obtained evidence supporting this hypothesis by restoring expression of TM1, one of two suppressed tropomyosins, in the v-Ki-ras transformed NIH3T3 cell line, DT, by retroviral mediated cDNA transfer. Cell clones expressing the cDNA had elevated levels of TM1 and lost the ability to grow under anchorage-independent conditions. They also did not participate in formation of tumors in athymic mice. However morphological reversion was incomplete. Elevated levels of TM1 synthesized in the transduced clones were only partially utilized in the cytoskeleton and disrupted microfilament bundles were only partially restored. Abnormal levels of TM1 homodimers and of crosslinked homodimers were produced which may associated poorly with the cytoskeleton. Studies with double insertion of both TM1 and TM2 suggest that under these conditions complete reversion of the transformed phenotype may occur.

CRADA Partner: THE DOW CHEMICAL COMPANY CRADA Number: CACR 0014

Title: "Generation and Characterization of Monoclonal Antibody-Radionuclide Conjugates for the Treatment of Human Carcinoma"

CRADA Initiation Date: 2/1/87 Amended 1/13/93

Objectives: The Laboratory of Tumor Immunology and Biology has developed a series of monoclonal antibodies (MAbs) that are reactive with a range of human carcinomas. The Dow Chemical Company has generated radionuclides from rare earth metals and has developed proprietary chelates to couple the isotopes to MAbs. The Laboratory of Tumor Immunology and Biology and The Dow Chemical Company are collaboratively developing recombinant humanized forms of various MAbs, and are conducting tumor targeting trials with these MAbs. The Dow Chemical Company is producing clinical grade murine and chimeric antibody as well as antibody-chelate-isotope reagents for patient administration. The Laboratory of Tumor Immunology and Biology (National Cancer Institute) is conducting Phase I and Phase II clinical trials to determine the safety and efficacy of MAb-radionuclide conjugates for radioimmunoguided surgery and radioimmunotherapy of human carcinoma.

Significance: This project is of importance because it may lead to the development of novel therapeutics for a range of human carcinomas.

CRADA Partner: ELI LILLY AND COMPANY

CRADA Number: CACR 0120

Title: "Development of Monoclonal Antibody-Drug Conjugates for Cancer Therapy"

CRADA Initiation Date: 3/16/92

Objectives: The Laboratory of Tumor Immunology and Biology has developed a series of monoclonal antibodies (MAbs) that are reactive with a range of human carcinomas. Eli Lilly and Company has developed proprietary linker technology for the conjugation of oncolytic drugs to MAbs for the development of novel MAb-drug conjugates for the therapy of human carcinomas. The Laboratory of Tumor Immunology and Biology is characterizing the biology of the antigen-antibody systems, developing recombinant humanized forms of various MAbs, and is conducting tumor targeting trials with these MAbs. Eli Lilly and Company is optimizing novel linker technologies with selected oncolytic drugs and conducting pre-clinical experimental evaluations of the conjugates for anti-tumor activity. The Laboratory of Tumor Immunology and Biology (National Cancer Institute) and Eli Lilly and Company plan to conduct clinical trials on a collaborative basis to determine the safety and efficacy of MAb-drug conjugates for therapy of human carcinoma.

Significance: This project is of importance because it may lead to the development of novel therapeutics for a range of human carcinomas.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CB 05190-13 LTIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## 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)

PI:	Jeffrey Schlom	Chief	LTIB, DCBDC, NCI
Others:	Margaret Schott	Senior Staff Fellow	LTIB, DCBDC, NCI
	Diane Milenic	Microbiologist	LTIB, DCBDC, NCI
	Patricia Horan Hand	Research Chemist	LTIB, DCBDC, NCI
	Diane Eggenesperger	Senior Staff Fellow	LTIB, DCBDC, NCI

Continued on page 2

## COOPERATING UNITS (if any)

Dr. F. Guadagni, Regina Elena Cancer Institute, Rome, Italy; Dr. W. Goekeler, The Dow Chemical Co. Midland, MI; Dr. D. Johnson, Eli Lilly and Co. Indianapolis, IN; Dr. M. Whitlow, Enzon, Gaithersburg, MD; Dr. S. Creekmore, BRMP, NCI; Drs. J. Greenblat, D. Parkinson, CTEP, NCI; Continued on page 2

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Experimental Oncology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 9.5

PROFESSIONAL: 3.1

OTHER: 6.4

## 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.)

Progress has been made in the characterization of monoclonal antibodies (MAbs) to three carcinoma associated antigens and the potential use of these MAbs in both the diagnosis and therapy of a range of carcinomas. The three antigens are (a) TAG-72, a high molecular weight mucin expressed in gastrointestinal, breast, ovarian, endometrial, prostate and non-small cell lung cancers, which is recognized by MAbs B72.3, CC49 and CC83; (b) carcinoembryonic antigen (CEA) a 180,000 D gp, expressed in gastrointestinal, breast and non-small cell lung cancer, which is recognized by MAbs COL-1 through 15 and (c) a 48,000 D gp expressed on colon carcinomas and normal colon, which is recognized by MAb D612. (I) To test the hypothesis of whether high affinity MAbs have a greater therapeutic efficacy, the anti-tumor activity of three radiolabeled anti-TAG-72 MAbs in a human colon cancer xenograft model was analyzed; MAbs CC49 and CC83 have a 8-fold and 10-fold higher affinity than B72.3, respectively. At all doses examined, the higher affinity MAbs demonstrated greater anti-tumor effects. A novel radioimmunoconjugate, lutetium (<sup>177</sup>Lu)-labeled CC49 was analyzed in this same experimental model. <sup>177</sup>Lu is a rare earth lanthanide with unique radiopharmaceutical properties. Strong anti-tumor effects were observed with minimal toxicity. A single chain Fv has been constructed from MAb CC49 and has shown good tumor targeting properties, and rapid plasma and whole body clearance in a xenograft model. Quantitative autoradiographic analyses revealed that penetration through tumors with the sFv was extremely rapid and more homogeneous as compared to intact IgG. It has also been determined that a <sup>177</sup>Lu-labeled sFv had a much different metabolic profile than iodinated sFv CC49. These studies have implications in the design of potential clinical trials. Employing the CA72-4 immunoassay to detect the TAG-72 antigen in the serum of cancer patients, it was shown that combined analyses of TAG-72 and the 19-9 antigen levels significantly increased the identification of patients with gastric cancer, while combined use of assays to detect TAG-72 and CEA increased positive rates for colorectal cancer patients, in both cases with no substantial increase in false positive. (II) Using a series of cell lines transduced with the CEA and related genes as controls, it was shown that in addition to GI carcinomas, CEA is expressed in approximately 50% of human breast cancers and 70% of non-small cell lung cancers. Further evaluation of the anti-CEA MAb COL-1 revealed that <sup>131</sup>I-COL-1 could efficiently target and eliminate the growth of established human tumors in a xenograft model. (III) The antigen recognized by MAb D612 was further characterized. (IV) Numerous collaborative Phase I and II clinical trials with MAbs CC49, COL-1 and D612 have been completed or are in progress.

**Professional Personnel Continued:**

Kwong Tsang	Expert	LTIB, DCBDC, NCI
Yomie Chung	Senior Staff Fellow	LTIB, DCBDC, NCI
Cheng-Feng Qi	Visiting Fellow	LTIB, DCBDC, NCI
Rosaria Dellipipi	Visiting Fellow	LTIB, DCBDC, NCI

**Cooperating Units Continued:**

Dr. J. Carrasquillo, NM, NCI; Dr. J. O'Shaughnessy, DCT, COP, NCI.

**Major Findings**

Progress has been made in the characterization of MABs to three carcinoma associated antigens and their potential use in both the diagnosis and therapy of a range of carcinomas. The three antigens are (i) TAG-72, a high molecular weight mucin expressed in gastrointestinal, breast, ovarian, endometrial, prostate and non small cell lung cancers, which is recognized by MABs B72.3, CC49 and CC83; (ii) carcinoembryonic antigen (CEA) a 180,000d gp, expressed in gastrointestinal, breast, and non small cell lung cancers, which is recognized by MABs COL 1 thru 15, and (iii) a 48,000 d gp expressed on colon carcinomas and normal colon mucosa which is recognized by MAB D612.

**MABs to TAG-72.** The effect of the relative affinity ( $K_A$ ) on the antitumor efficacy of MABs has been questioned. We have previously shown in experimental models that the use of MABs with higher relative affinities results in a higher percentage of injected dose of MAB bound to tumor. On the other hand, mathematical models have proposed that the use of higher affinity MABs may be disadvantageous for antitumor effects, since higher affinity MABs could bind more antigen and prevent penetration of MAB through tumor. To test this hypothesis, three MABs reacting with TAG-72 were used as radioimmunoconjugates for therapeutic efficacy. MABs B72.3, CC49, and CC83 have all been shown by depletion studies to react to the same molecule and to react with overlapping epitopes. While the relative  $K_A$  of B72.3 is  $2.5 \times 10^9 M^{-1}$ , the relative  $K_A$ s of CC49 and CC83 are  $16.2$  and  $27.7 \times 10^9 M^{-1}$ , respectively. Each MAB was radiolabeled with  $^{131}I$ , and each radioimmunoconjugate was assayed at five dose levels for therapeutic efficacy using the human xenograft model. The results demonstrated substantial therapeutic advantage of the higher affinity MABs CC49 and CC83 versus B72.3 at every dose level.

We have also undertaken collaborative studies to develop novel radioimmunotherapeutics for the treatment of carcinoma. As part of these studies, lutetium-177 ( $^{177}Lu$ ) was used with the bifunctional chelate PA-DOTA to radiolabel MAB CC49. Lutetium is a member of the family of elements known as lanthanides or rare earths. These studies constituted the first employing a  $^{177}Lu$ -immunoconjugate.  $^{177}Lu$ -CC49 was shown to delay the growth of established LS-174T human colon carcinomas in athymic mice. Dose fractionation experiments revealed that at least  $750 \mu Ci$  of  $^{177}Lu$ -CC49 ( $250 \mu Ci/week$  for 3 consecutive weeks) was well tolerated. Moreover, this dose schedule was able to eliminate the growth of relatively large human colon tumor xenografts. We have now analyzed other heavy metal radionuclides for potential use in immunotherapy. Like lutetium-177, yttrium-90 and samarium-153 are members of the lanthanides or rare earth family of elements. We have defined the comparative biodistributions of CC49 IgG and CC49 F(ab')<sub>2</sub> fragments, when labeled with  $^{90}Y$ ,  $^{153}Sm$  or  $^{177}Lu$ , using the bifunctional chelating agent PA-DOTA. Chelation and conjugations gave similar yields and the labeled proteins showed similar immunoreactivities regardless of the radioisotope used for both the whole antibodies and the fragments. Likewise, biodistribution studies carried out in athymic mice bearing xenografts showed no differences between the three radioisotopes for both the whole antibodies and the fragments. These studies demonstrated that a variety of radiolanthanides can be attached to proteins via the bifunctional chelating agent PA-DOTA, and

that their biodistributions are similar. This flexibility in radioisotope selection using a common chelation chemistry should allow for the design of novel radiotherapeutics, where the pharmacokinetic properties of the MAb or genetically modified MAb are compatible with the decay properties of the radionuclide for the specific therapeutic application.

We have conducted studies on the potential utility of single chain Fv molecules to target tumors in an experimental xenograft model. These studies were conducted with an sFv of MAb CC49. This molecule, which was constructed and expressed in *E. coli*, is a recombinant protein composed of a V<sub>L</sub> amino acid sequence of an immunoglobulin tethered to a V<sub>H</sub> sequence by a designed peptide. The CC49 sFv was shown to be a M<sub>r</sub> 27,000 homogeneous entity which could be efficiently radiolabeled with <sup>125</sup>I or <sup>131</sup>I. Comparative direct binding studies and competition radioimmunoassays using CC49 intact IgG, F(ab')<sub>2</sub>, Fab', and sFv revealed that the monomeric CC49 Fab' and sFv have relative binding affinities 8-fold lower than the dimeric F(ab')<sub>2</sub> and intact IgG. Tumor targeting studies with all four radiolabeled Ig CC49 forms, using the LS-174T human colon carcinoma xenograft model, revealed a much lower %ID/g tumor binding for the CC49 monomeric sFv and Fab' as compared to the dimeric F(ab')<sub>2</sub> and intact IgG. However, tumor:normal tissue ratios (RIs) for the sFv were comparable to or greater than those of the other Ig forms. High kidney uptake with <sup>125</sup>I-labeled Fab' and F(ab')<sub>2</sub> was not seen with <sup>125</sup>I-sFv. One of the issues we raised in the analysis of the iodinated sFv metabolic studies, however, was whether similar metabolic patterns would be observed if the sFv were labeled with a radiometal. <sup>125</sup>I-CC49 sFv and <sup>177</sup>Lu-CC49 sFv were co-injected in mice bearing antigen-positive carcinoma xenografts. Both sFv forms showed similar tumor targeting and plasma clearance pharmacokinetics. The <sup>177</sup>Lu-sFv, however, showed a greater uptake in liver and spleen and a much higher uptake in kidney. These studies thus demonstrated that despite their small size, the metal-chelated sFv showed a metabolic pattern very different than that of the iodinated sFv, which is most likely due to retention of the metal by organs metabolizing the sFv. It thus appears that radiometal-chelated sFv conjugates may have limited use in cancer detection and/or therapy, although detection of tumor masses outside the liver and the kidneys may be possible. On the other hand, these studies provide further evidence for the potential utility of <sup>125</sup>I-labeled sFvs for tumor detection using the intraoperative hand-held probe, and <sup>131</sup>I (or <sup>123</sup>I)-labeled sFvs using gamma scanning. In both cases, the diagnostic procedures would be greatly shortened. Single chain antigen binding proteins, or sFvs, represent potentially unique molecules for targeted delivery of drugs, toxins, or certain radionuclides to a tumor site. One potential consequence of the rapid sFv pharmacokinetic properties was the reduced %ID/g of the radiolabeled sFv found in the tumor throughout a range of time points. A recent study was designed to define the tumor penetration properties of a radiolabeled sFv in comparison with other Ig forms. <sup>125</sup>I-labeled sFv, Fab', F(ab')<sub>2</sub>, and IgG forms of MAb CC49 were used to target the LS-174T human colon carcinoma xenograft. At various time points, after systemic Ig administration, quantitative autoradiographic analyses of surgically removed tumors were used to define the rate and degree of penetration of the various Ig forms. These studies revealed that most of the intact IgG delivered to the tumor was concentrated in the region of or immediately adjacent to vessels, while the sFv was more evenly distributed throughout the tumor mass. These studies thus reveal a greater degree of uptake throughout the tumor for the sFv than would be expected by gross analyses of %ID/g.

We have collaboratively developed a more efficient assay for the detection of TAG-72 in serum using both MAb B72.3 and the second generation MAb CC49 (designated CA72-4). Using this assay, only 3.5% of normal sera and 6.7% of sera from patients with benign gastrointestinal diseases had TAG-72 levels greater than 6 U/ml. In a study to define the potential use of the CA72-4 assay in the management of gastric cancer, the presence of three distinct serum markers of carcinoma, TAG-72 (as measured by the CA72-4 assay), CA19-9, and CEA, was evaluated in 194 patients diagnosed with either malignant or benign gastric disease. The data indicated that the measurement of TAG-72 with CA19-9 significantly increased the percentage of gastric carcinoma patients with positive serum levels of either antigen. This advantage was achieved

with a minimal increase in the number of false positives. We also evaluated serum TAG-72, CEA and CA19-9 in 300 patients diagnosed with either malignant or benign colorectal carcinoma. The measurement of TAG-72 in combination with CEA, for patients with primary or recurrent colorectal carcinoma substantially increased the percentage of the serum samples which were positive, when compared with measuring each serum tumor marker alone. This was achieved with little increase in the number of false positives. In a longitudinal study, 82 patients diagnosed with gastrointestinal (GI) adenocarcinoma were evaluated before and for 26 months after primary tumor resection for the presence of TAG-72 and CEA. The elevation of one or both markers correlated with the clinical status in 10 of 11 patients with recurrence. In addition, 20 patients who were clinically free of disease after more than 700 days' follow-up had normal serum levels of both TAG-72 and CEA. These preliminary findings suggest that the combined use of serum TAG-72 and CEA measurements may improve detection of recurrence in patients with GI cancer and may be useful in the postsurgical management of GI adenocarcinoma patients.

Several collaborative Phase I and II clinical trials involving  $^{131}\text{I}$ ,  $^{125}\text{I}$  and  $^{177}\text{Lu}$ -labeled MAb CC49 are now in progress in colorectal, breast, lung, and ovarian cancer patients.

**MABs to D612.** MAb D612 has previously shown to efficiently mediate ADCC of human colon carcinoma cells using human effector cells. We have carried out studies to define and characterize the colon associated antigen detected by MAb D612. SDS-PAGE analyses of MAb D612 immunoprecipitates of human colon carcinoma cells showed that the D612 antigen is a  $M_r$  48,000 glycoprotein. The D612 antigen was shown to be clearly distinct from previously described gastrointestinal carcinoma-associated glycoproteins. SDS-PAGE analyses of the D612 antigen radiolabeled in the presence of tunicamycin demonstrated that the MAb D612-reactive epitope resides on the  $M_r$  42,000 polypeptide core of the antigen. It has been determined that the number of binding sites for MAb D612 on the LS-174T human colorectal carcinoma cell line is  $4.8 \times 10^5$ . MAb D612 was found to have a relative  $K_a$  of  $1.3 \times 10^9 \text{M}^{-1}$ . Collaborative Phase I and II trials with unconjugated MAb D612 in colorectal cancer patients are currently underway.

**MABs to CEA.** To further evaluate the potential use of the anti-CEA MAb COL-1, or for that matter any potential anti-CEA MAb, we took advantage of the fact that we had transduced the human CEA gene and the CEA-related genes NCA (normal cross reacting antigen) and BGP (biliary glycoprotein) into murine cells. These were used to analyze the specificity of several MABs. The MABs COL-1 and COL-6 were shown to react with the murine cells transfected with CEA but not with the same cells transfected with the NCA or BGP gene. The COL-1 and COL-6 MABs were then utilized in the histochemical analysis of a number of primary and secondary breast and lung carcinomas as well as colon carcinomas. The results showed that approximately 50% of breast carcinomas, and 70% of non-small cell lung carcinomas express CEA; fairly homogeneous expression of CEA was seen. Our results thus indicate that CEA may be an important target for immunotherapy in a number of patients with breast and lung carcinomas in addition to GI carcinomas.

Our biochemical and histochemical studies with a series of the COL MABs have led us to choose MAb COL-1 for further experimental and clinical studies.  $^{125}\text{I}$ -labeled COL-1 IgG was shown to efficiently and specifically target the LS-174T human colon carcinoma xenograft in athymic mice. Dose titration studies in this same model with  $^{131}\text{I}$ -labeled COL-1 demonstrated reduction of tumor growth rate when  $300\mu\text{Ci}$  of the immunoconjugate was used. Dose fractionation experiments with  $^{131}\text{I}$ -COL-1 demonstrated the ability to administer much higher levels of the immunoconjugate with little or no toxicity, which resulted in a greater therapeutic efficacy. These results indicated a potential therapeutic use for radiolabeled COL-1 in clinical trials and further demonstrate the principle of the advantage of dose fractionation protocols for immunoconjugates. A collaborative Phase I study using  $^{131}\text{I}$ -labeled MAB COL-1 in colorectal cancer patients is underway.

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

PROJECT NUMBER  
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PERIOD COVERED

October 1, 1992 to September 30, 1993

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)

PI:	John Greiner	Chemist	LTIB, DCBDC, NCI
Others:	Claudio Dansky Ullmann	Associate	LTIB, DCBDC, NCI
	Carol Nieroda	Senior Staff Fellow	LTIB, DCBDC, NCI
	Joanne McLaughlin	Biotech Fellow	LTIB, DCBDC, NCI
	Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)

Dr. S. Pestka, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ;  
 Dr. E. Borden, Medical School of Wisconsin, Milwaukee, WI

LAB/BRANCH Laboratory of Tumor Immunology and Biology

SECTION Experimental Oncology Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 6.1	PROFESSIONAL: 4.5	OTHER: 1.6
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<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		A

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

Tumor antigen heterogeneity may at times impact on monoclonal antibody (MAB) targeting, thereby compromising its overall effects as an immunodiagnostic and/or immunotherapeutic agent. This area of research involves the study of cytokines which up-regulate human tumor antigen expression. We have reported that the recombinant interferons can selectively enhance the expression of carcinoembryonic antigen (CEA), tumor-associated glycoprotein-72 (TAG-72) and a potentially novel Mr 110,000 antigen in a variety of human carcinomas. In a preliminary study, interleukin-6 treatment of human colon carcinoma cells was shown to also augment the level of expression of CEA and class I HLA. Interferon-gamma (IFN- $\gamma$ ), in particular, substantially increases TAG-72 and CEA expression in human tumor xenografts leading to an increased tumor MAb localization. The ability of IFN- $\gamma$  to enhance MAB targeting to the tumor site provided a significant therapeutic advantage over the MAB administration alone. Those preclinical findings provided the rationale to investigate the ability of interferon to augment human tumor antigen expression in a clinical setting. Patients diagnosed with either ovarian or gastrointestinal carcinoma with secondary ascites were given weekly intraperitoneal doses of IFN- $\gamma$  (0.1 to 100 MU). Analyses of TAG-72 and CEA expression on isolated malignant ascites cells showed a dramatic increase in the expression of both tumor antigens as a result of IFN- $\gamma$  treatment. In some cases, IFN- $\gamma$  increased the percentage of tumor cells expressing either TAG-72 or CEA from 10% to >90% and those increases were observed at low IFN- $\gamma$  doses (ie, 0.1-1.0 MU) which were well tolerated by all patients. Thus, both experimental and early clinical results provide substantial support for the use of a cytokine as an adjuvant to enhance MAB localization to human carcinoma cell populations. Those changes may substantially improve MAB-based strategies designed for the diagnosis and treatment of human cancer.

### Major Findings

In a previous report, we identified a  $M_r$  110,000 antigen which shared some similarities with carcinoembryonic antigen (CEA) and other members of that gene family. Reactivity of monoclonal antibodies (MAb), B1.1 and COL-4, with the  $M_r$  110,000 antigen indicated the presence of at least two binding epitopes which were previously shown to be on CEA and related antigens. Six of eight human gastric carcinoma cell lines constitutively expressed the  $M_r$  110,000 antigen. One gastric tumor cell line, Hs746T, expressed the  $M_r$  110,000 antigen, but not CEA nor NCA, indicating distinct regulatory pathways for the  $M_r$  110,000 antigen. Northern blot analyses using either specific or broadly-reactive CEA and NCA complementary DNA (cDNA) probes did not identify any transcripts in poly(A)<sup>+</sup>-selected mRNA isolated from the Hs746T cells. Finally, primers that amplify the 420 base pairs of the immunoglobulin-like domain of CEA and NCA detected an appropriately sized product in human gastric carcinoma cells using the polymerase chain reaction (PCR) method. Previous studies had shown that CEA and related antigens were attached to the cell membrane by a glycosyl-phosphatidylinositol anchor. That linkage could be cleaved, thus releasing the antigens from the cell membrane, with phosphatidylinositol phospholipase C (PI-PLC) treatment. We found that PI-PLC treatment did not release the  $M_r$  110,000 antigen from its membrane attachment, suggesting that membrane attachment of this novel antigen is not via a glycosyl-phosphatidylinositol anchor. Thus, a potentially novel gene encoding for a  $M_r$  110,000 antigen has been identified in human gastric carcinoma cells. Immunologically the antigen shares reactive epitopes with CEA and its related NCA gene product; however, Northern blot analyses, PCR, and PI-PLC results suggest that the antigen may be, at best, a distant relative of the CEA gene family.

Concomitant studies also revealed that interferon- $\gamma$  (IFN- $\gamma$ ) treatment substantially increased the level of expression of the  $M_r$  110,000 antigen on six of the eight gastric tumor cell types that constitutively express the antigen. IFN- $\gamma$  treatment also *de novo* induced the expression of the  $M_r$  110,000 antigen on the surface of GaCa gastric carcinoma cells. The regulation by IFN- $\gamma$  of the expression of  $M_r$  110,000 antigen was compared with that of CEA, NCA, CA19-9, 17-1A, TAG-72, and an  $M_r$  48,000 antigen reactive with MAb D612 in eight human gastric cancer cell lines. Six of the seven tumor antigens have been well-characterized and reported to be expressed by human gastric carcinomas [one exception - the D612-reactive  $M_r$  48,000 antigen]. IFN- $\gamma$  administration substantially increased the expression of the  $M_r$  110,000 antigen in six gastric tumor cell types, and, furthermore, induced its *de novo* expression in another gastric tumor cell line (GaCa). Constitutive CEA and NCA expression was detected on the surface of five of the eight gastric carcinoma cell lines. IFN- $\gamma$  treatment induced only a modest increase in the level of expression of those antigens, and those changes were accompanied by comparable increases in the level of the respective mRNA transcripts. Four other human tumor-associated antigens, TAG-72, CA19-9, D612, and 17-1A, were found either to be not constitutively expressed or their constitutive level of expression not enhanced as a result of IFN- $\gamma$  treatment. The results indicate the selective nature by which IFN- $\gamma$  regulates the  $M_r$  110,000 antigen and, to a lesser extent, the antigens of the CEA gene family in human gastric carcinoma cells.

Later we described the molecular cloning and sequence analyses of overlapping clones which constitute a full length complementary DNA which encodes for the entire  $M_r$  110,000 molecule. The 1.5 kb message encodes for a 407 amino acid polypeptide whose structural analysis was consistent with an integral membrane glycoprotein. In particular, the extracellular domain was rich in serine and threonine residues at which carbohydrate substitution is likely through O-linked glycosylation. This would explain the higher molecular weight of the antigen whose polypeptide backbone is approximately 42 kD. Further computer-aided sequence analyses revealed no significant homology with any member of the CEA gene family. The cross-reactivity with anti-CEA MAbs seems to be explained by the presence of CEA and NCA homologous amino acid sequences proximal to the transmembrane region. No sequence homology was found with any



known protein. Thus, the  $M_r$  110,000 molecule represents a potentially novel cell membrane glycoprotein whose possible role(s) in human gastric cancer and/or as a IFN- $\gamma$ -inducible gene product warrants subsequent investigation.

In our earlier studies, it was apparent that tumor antigen expression was not increased by interferon treatment in all human tumor cells. We characterized a resistant human colon tumor cell line which showed no change in either TAG-72, CEA or HLA expression levels after interferon treatment. Those observations led to a series of studies which evaluated other types of differentiation-inducing agents for their ability to augment surface antigen expression. It was reasoned that other cellular pathways may also lead to enhancement of surface tumor and normal antigen expression. We reported that analogues of cyclic AMP increased CEA expression using a human colon carcinoma cell line previously shown to be resistant to interferon. Those findings implicated a protein kinase pathway mediating the changes in CEA expression. The interleukins have been primarily involved in the stimulation of proliferation and differentiation of cytotoxic T cells. We became interested in whether those cytokines could also elicit any change in the antigen expression on the target tumor cells. Therefore, a series of human recombinant interleukins (IL-1, -2, -4, -6, -7, -8) and GM-CSF were analyzed for their ability to alter the level of CEA and MHC antigen expression on the surface of human colorectal tumor cells. IL-6 was the only interleukin which could increase the level of CEA and HLA class I expression on the surface of established human colon tumor cell lines. Human colorectal carcinoma cells which were treated *in vitro* with IL-6 expressed increased cell surface levels of CEA and normal HLA class I antigens. The IL-6-mediated increase of CEA expression on the surface of a moderately differentiated colon carcinoma cell line [WiDr] was time- and dose- dependent. A five day treatment of the WiDr cells with 100 units IL-6/ml increase the percentage of WiDr cells which expressed CEA from 29% to >70% and also enhanced the level of HLA class I expression. The change induced in CEA expression was also observed using SDS-PAGE/Western blot analysis and subsequent Northern blot analysis revealed concomitant increases in CEA-related mRNA transcripts with IL-6 treatment. Comparing the changes in CEA expression after IL-6 treatment with those induced by interferon- $\beta_{\text{ser}}$  and  $\gamma$  revealed similar potencies on a unit protein basis. Thus, IL-6 can selectively increase CEA and HLA class I expression on the surface of human colon carcinoma cells which may provide some insight into the mechanisms by which IL-6 can inhibit tumor cell growth by enhancing tumor cell recognition and rendering the cell more sensitive to antibody-dependent and/or cell mediated cytotoxic mechanisms.

A considerable amount of early clinical data have been generated concerning the tumor targeting ability of different anti-TAG-72 MABs. Results clearly showed the localization of MABs B72.3 and CC49 to primary as well as metastatic malignant lesions with minimal uptake by normal tissues. Those findings also revealed that tumor localization of those anti-TAG-72 MABs was highly correlated with tumor antigen content [i.e., the higher levels of TAG-72, the better the localization]. Those results provided the rationale to investigate whether the addition of an antigen augmentation protocol using interferon administration could improve MAB tumor targeting and associated antitumor effects in an experimental animal model. TAG-72 regulation by the interferon has been difficult to evaluate due to the lack of established cell lines which constitutively express the antigen. However, using human carcinoma cells isolated from malignant effusions, the administration of either type I or type II interferon *in vitro* resulted in an increase in TAG-72 expression in >75% of the cases. Initial *in vivo* studies established that the TAG-72-positive LS174T cells were unresponsive to antigen augmentation following IFN- $\gamma$  administration. Subsequent investigations, however, revealed that the moderately differentiated human colon tumor cell line, HT-29, constitutively expresses low levels TAG-72 as well as CEA when grown as subcutaneous tumors in athymic mice. Moreover, *in vivo* IFN- $\gamma$  administration increased both TAG-72 and CEA levels in the HT-29 tumor xenografts in a time- and dose-dependent manner. Immunohistochemical staining revealed a more homogeneous TAG-72-positive tumor cell population after IFN- $\gamma$  which accounted for an enhanced localization of anti-TAG-72 [i.e., CC49] MABs to the HT-29 tumors. Using that experimental model, subsequent

studies presented evidence showing that the combination of IFN- $\gamma$  with  $^{131}\text{I}$ -CC49, resulted in a statistically significant improvement in the therapeutic efficacy when compared with  $^{131}\text{I}$ -CC49 alone. For example, treatment with 300  $\mu\text{Ci}$  of  $^{131}\text{I}$ -CC49 initially suppressed HT-29 tumor growth, however, that reduction in tumor growth was transient as evidenced by the emergence of tumor growth at later time points. An 8-day treatment of IFN- $\gamma$  in combination with 300  $\mu\text{Ci}$   $^{131}\text{I}$ -CC49 induced a sustained suppression of HT-29 tumor growth. In fact, the combination therapy resulted in no palpable tumors in 33% of the mice. In a subsequent study, well-established HT-29 tumors were treated with two separate regimens of 300  $\mu\text{Ci}$  CC49 alone or in combination with IFN- $\gamma$ . Those results also indicated that the combination of IFN- $\gamma$  with  $^{131}\text{I}$ -CC49 led to a statistically significant improvement in antitumor effects when compared with the administration of  $^{131}\text{I}$ -CC49 alone. In some cases, multiple treatments of  $^{131}\text{I}$ -CC49 and IFN- $\gamma$  could significantly shrink 200-250 mg HT-29 tumors in the athymic mice. It should be noted that those tumors were approximately 1.0% of the weight of a 20 g athymic mouse. Thus, IFN- $\gamma$  *in vivo* can substantially increase the TAG-72 expression in human colon tumor xenografts which leads to an increased tumor localization of anti-TAG-72 MABs, thus enhancing the antitumor effects when IFN- $\gamma$  was combined with  $^{131}\text{I}$ -CC49. The data support further investigations to determine whether combining a radiolabeled MAB with IFN- $\gamma$  might have some therapeutic benefit in an adjuvant setting in patients with minimal residual disease. Moreover, the ability to substantially reduce the size of large well-established HT-29 tumors leads one to hypothesize that this same combination may be effective for the treatment of primary and/or recurrent colorectal carcinoma.

The aforementioned experimental models clearly indicated that the ability of interferon to enhance TAG-72 and/or CEA expression could be exploited resulting in an improved antitumor effects of radionuclide-conjugated MABs. In an earlier clinical study, investigators reported the increased tumor localization of an antimelanoma MAB in patients administered IFN- $\alpha$ . However, neither that, or any other study, showed that the administration of interferon could, indeed, augment the expression of tumor antigens on human carcinoma cells. In collaboration with investigators at the University of Wisconsin, a phase 1A clinical trial was designed to investigate the ability of IFN- $\gamma$  to enhance TAG-72 and/or CEA expression. Eight patients diagnosed with adenocarcinoma (six ovarian, two gastrointestinal) with secondary malignant ascites were administered escalating doses of IFN- $\gamma$  (i.e., 0.1-100 MU) intraperitoneally (i.p.) each week for 8 weeks. The i.p. administration of escalating doses of IFN- $\gamma$  resulted in ascites IFN- $\gamma$  levels that ranged from 1.0 to >1,200 U/mL at 24 hr after treatment. Peak IFN- $\gamma$  levels in the ascites fluid confirmed the pharmacokinetic advantage of delivering the cytokine by the intracavitary route. Ascites samples, prior to and 24 and 48 hr post-IFN- $\gamma$ , were removed each week, tumor cells isolated, and the level of TAG-72 and CEA measured using immunocytochemistry and flow cytometry. I.p. IFN- $\gamma$  dramatically increased TAG-72, measured by B72.3 and CC49 reactivity, as well as CEA expression, measured by COL-1 reactivity on carcinoma cells isolated from the malignant ascites. Enhanced TAG-72 expression on carcinoma cells was observed during the initial week of IFN- $\gamma$  and throughout the treatment schedule. Twenty-four hrs after the administration of 0.1 MU IFN- $\gamma$ , 1-2 U/mL of the cytokine was found in the ascites, while no detectable IFN- $\gamma$  was measured in the sera. Yet, TAG-72 expression was increased in all seven patients during the first week of treatment. The most dramatic change in TAG-72 expression occurred during the first 4 weeks of treatment - dose range of 0.1 to 1.0 MU. In some cases, the percentage of tumor cells reactive with MAB B72.3 or CC49 was increased from 10% to >90%. Malignant ascites cells isolated from two patients diagnosed with gastrointestinal carcinoma expressed increased levels of CEA following i.p. IFN- $\gamma$ . The pharmacokinetic advantage gained by the intracavitary delivery of IFN- $\gamma$  raises the question as to whether systemic administration could effectively up-regulate tumor antigen expression on primary and metastatic carcinomas. Recently, an optimum immunomodulatory dose of a different IFN- $\gamma$  preparation was described in patients with malignant melanoma. Several IFN- $\gamma$ -sensitive biological parameters, such as Fc receptor and HLA-DR expression on monocytes, NK cell activity, etc., were increased after the intramuscular or subcutaneous administration of 0.1 mg IFN

$\gamma/m^2$ . HLA-DR expression was maximally induced at IFN- $\gamma$  doses well below 0.1 mg/m<sup>2</sup>. Our experimental data suggests that *de novo* expression of HLA-DR is approximately 5- to 10-fold more sensitive than are changes induced in CEA expression by IFN- $\gamma$ . Additional studies are currently underway to establish whether the systemic administration of IFN- $\gamma$  or IFN- $\alpha$  could enhance tumor as well as normal antigen expression in human carcinoma tissues in order to further substantiate this approach to improve the effectiveness of MAbs in immunoscintigraphy and immunotherapy.

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

PROJECT NUMBER  
 ZO1 CB 09023-07 LTIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Cloning and Modification of Anti-Tumor Antigen Immunoglobulin Genes

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

PI: Syed Kashmiri	Expert	LTIB, DCBDC, NCI
Others: Patricia Horan Hand	Research Chemist	LTIB, DCBDC, NCI
Liming Shu	Visiting Associate	LTIB, DCBDC, NCI
William Shupert	BTP Fellow	LTIB, DCBDC, NCI
Hyun-Sil Lee	Visiting Fellow	LTIB, DCBDC, NCI

Continued on page 2

COOPERATING UNITS (if any)

E. Padlan, NIDDK, NIH; K. Anderson, The Dow Chemical Co., Midland, MI; D. Johnson, Eli Lilly, Indianapolis, IN.

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 7.2

PROFESSIONAL: 4.2

OTHER: 3.0

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 major goal of this research effort is to design, construct and generate novel recombinant immunological reagents for the diagnosis and therapy of human cancers. Several hybridoma cell lines have been developed in this laboratory that produce monoclonal antibodies (MAbs) selectively reactive with carcinoma associated antigens. These include MAbs against carcinoembryonic antigen (CEA), tumor associated glycoprotein (TAG)-72, a high-molecular weight mucin present on a variety of carcinomas, and Mab D612, which is reactive with a 48 kD antigen expressed on the surface of normal and malignant gastrointestinal epithelium. In ongoing clinical trials, the anti-TAG-72 MAbs, B72.3 and CC49, D612 and an anti-CEA Mab, COL-1, have shown various degrees of potential for being developed into diagnostic and therapeutic reagents. However, the usefulness of murine MAbs for in vivo diagnosis and therapy is limited because of their immunogenicity. To reduce this potential problem we have developed mouse-human chimeric (c) MAbs, including cB72.3 ( $\gamma$ 1), using recombinant DNA techniques. In an effort to optimize the pharmacokinetics of plasma clearance and to maximize the efficiency of localization of, and penetration into, tumors we have developed novel chimeric immunoglobulin variants; these include aglycosylated cB72.3 ( $\gamma$ 1) Mab and constant region domain-deleted variants of cB72.3 ( $\gamma$ 1). In comparison with the cB72.3 Mab, the CH2 domain-deleted cMab demonstrated a faster clearance rate and a more rapid tumor targeting. For second generation anti-TAG-72 MAbs, the heavy and light chain genes of Mab CC49 and CC83 have been cloned, sequenced and inserted into retroviral vectors. Recently, the cCC49 ( $\gamma$ 1) has been expressed and purified. We have also developed a single gene encoded Ig molecule which has retained effector functions. We have also developed a cD612 Mab which has been expressed and secreted by a human T cell line. The secreted immunoglobulin retained its antigen-binding properties and its ability to mediate ADCC against human tumor cells. To our knowledge, this is the first demonstration of the production of an IgG by human T cells and opens the possibility of a therapeutic approach in which T-cells secrete humanized anti-tumor Mab capable of mediating ADCC at the tumor site.

**Professional Personnel continued**

Kwong Y. Tsang	Expert	LTIB, DCBDC, NCI
Rosaria DeFillippi	Visiting Fellow	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

**Major Findings:**

**Aglycosylated cB72.3 ( $\gamma$ 1).** It has been demonstrated that the degree of glycosylation of a molecule may alter its pharmacokinetic properties and, in the case of an antibody, its metabolism and other biological properties. We have developed an aglycosylated (aG) cB72.3 ( $\gamma$ 1). To generate this Ig, asparagine 297 of the glycosylation consensus sequence, Asn-Ser-Thr, of the heavy chain was changed to glutamine by site-specific mutagenesis. The subsequent expression construct was introduced into murine myeloma cells producing chimeric  $\kappa$  chains. Competition radioimmunoassays demonstrated that the aGcB72.3, secreted from the resulting transfectoma, and cB72.3 have comparable binding properties. SDS-PAGE profiles of the aGcB72.3 ( $\gamma$ 1) were consistent with the loss of the major glycosylation site of the Ig molecule at the CH2 domain. Aglycosylation eliminated antibody-dependent cell mediated cytotoxicity (ADCC) activity against TAG-72 positive KLE-B endometrial carcinoma cells. These studies also demonstrated for the first time (a) no difference in plasma clearance of the chimeric and aglycosylated chimeric versions of MAb B72.3 in primates after i.v. inoculation, (b) a difference ( $p < 0.05$ ) in mice in the more rapid peritoneal clearance of the chimeric compared to the aGcB72.3 MAb, and (c) higher ( $0.05 < p < 0.1$ ) tumor-to-liver ratios at 24, 72, and 168 hr using  $^{111}\text{In}$ -labeled aGcB72.3 versus cB72.3.

**Domain-Deleted Chimeric Immunoglobulins.** In an attempt to generate an Ig variant with a faster clearance rate, as well as more rapid tumor targeting than the intact murine or chimeric MAb, we have developed chimeric forms of B72.3 with constant region domain deletions. The domain-deleted antibodies were produced by generating mutants deficient in the CH2, CH3 or both domains, developing their expression constructs and subsequently introducing them into cells that produce chimeric kappa chains. Despite the absence of these domains, the transfectomas secrete H2L2 tetramers with appropriate antigenic specificity. The domain-deleted cMAbs can be purified by chromatography on Protein G Sepharose, which binds to a site on the Fab region of these Ig molecules. The CH2 domain-deleted Ig was chosen for further study. The pharmacokinetics of serum clearance of iodine-labeled MAbs cB72.3 $\Delta$ CH2 and the cB72.3 were compared in athymic mice; by 24 hr, less than 1% of the cB72.3 $\Delta$ CH2 was left in the plasma, while 36% of the cB72.3 still remained. Biodistribution studies in athymic mice bearing LS-174T xenografts showed a reduction in the percentage of injected dose per gram in tumor with  $^{131}\text{I}$ -cB72.3 $\Delta$ CH2; however, the  $^{131}\text{I}$ -cB72.3 $\Delta$ CH2 both localized to tumors faster and cleared from the blood faster than the  $^{125}\text{I}$ -cB72.3 MAb. Only trace amounts of the  $^{131}\text{I}$ -cB72.3 $\Delta$ CH2 were detected in normal tissues, including kidney. The faster clearance rate, more rapid tumor targeting and lack of metabolic uptake in normal tissues demonstrated with the iodine-labeled CH2 domain-deleted cMAb may be an advantage for certain clinical protocols.

**Chimeric MAbs of Second Generation anti-TAG-72 MAbs.** For developing cMAbs of second generation anti-TAG-72 antibodies, we have cDNA cloned and sequenced the heavy and light chain genes of the MAb CC49 and CC83. Using PCR amplification, the cDNA constructs encoding the mouse-human chimeric heavy ( $\gamma$ 1) and light chain ( $\kappa$ ) were generated and inserted into the retroviral vectors, pLHCX and pLNCX, respectively. The cCC49 ( $\gamma$ 1) molecule has been expressed and purified.

**A Single Gene Encoded Ig Molecule with Effector Functions.** We have recently designed a single gene, a DNA construct, that encodes a single chain protein consisting of the MAb CC49

VH (devoid of the CH1 domain) and VL (devoid of the CL domain) domains covalently joined through a short linker peptide (gly-ser), while the carboxyl end of the VL domain is linked to the amino terminal of the human  $\gamma$ 1 Fc region through the hinge region. This single chain has recently been shown to be expressed and secreted from mammalian cell transfectomas and is designated SCA $\Delta$ CLCH1. The native MAb CC49 and SCA $\Delta$ CLCH1 of CC49 showed similar binding properties to TAG-72, and the chimeric MAb CC49 and SCA $\Delta$ CLCH1 showed similar cytotoxic activity.

**Secretion of a Chimeric MAB from a Human T Cell Line.** Tumor-infiltrating lymphocytes (TILs) and MABs that can mediate ADCC via human effector cells have shown antitumor effects in both animal models and clinical trials. One potential novel approach would be to combine these two modalities in the creation of a T cell capable of secreting antitumor Ig at the tumor site. We have cloned the D612 MAB Ig genes and generated constructs encoding chimeric heavy and light chains of the MAB D612 containing the murine variable regions and human constant regions. The chimeric D612 was expressed and secreted by the human T cell line MOLT-4. The secreted Ig retained its antigen-binding properties and its ability to mediate ADCC against human tumor cells. To our knowledge, this is the first demonstration of the production of an IgG by human T-cells and opens the possibility of a therapeutic approach in which TILs secrete humanized anti-tumor MAB capable of mediating ADCC at the tumor site.

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Slavin-Chiorini D, Horan Hand P, Kashmiri SVS, Calvo B, Zaremba S, Schlom J. Pharmacological properties of a CH2 domain-deleted recombinant immunoglobulin. *Int J Cancer* 1992;53:1-7.

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

PROJECT NUMBER  
 ZO1 CB 09025-06 LTIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Cellular Immunotherapy of Human Carcinoma

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

<b>PI:</b> Kwong Y. Tsang	Expert	LTIB, DCBDC, NCI
<b>Others:</b> Rosaria DeFilippi	Visiting Fellow	LTIB, DCBDC, NCI
Cheng-Feng Qi	Visiting Fellow	LTIB, DCBDC, NCI
Hiroki Yamaue	Visiting Associate	LTIB, DCBDC, NCI
Syed Kashmiri	Expert	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)

Dr. John Yarelli, 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 STAFF YEARS: 2.7

PROFESSIONAL: 2.7

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.)

We have investigated the introduction and expression of the human IL-6 gene in human colorectal carcinoma cells. IL-6 secreted by the transfected HT-29 human colorectal carcinoma cells was shown to be biologically active. Significant enhancement in the expression of CEA, but not in the expression of HLA class I and class II and ICAM-1 antigens, was observed. These results thus suggest another potential role for the use of IL-6 gene transfer in the immunotherapy of human cancers. We also demonstrated an enhancement by recombinant human M-CSF (rhM-CSF) on the ADCC activity of human monocytes using several MABs. We have investigated the approach of combining tumor-infiltrating lymphocytes and anti-tumor MABs in the creation of a T cell capable of secreting anti-tumor Ig. The cDNA expression construct of the chimeric D612 heavy chain and light chain genes in retroviral vectors were introduced into MOLT-4 cells. The secreted Ig retained its antigen-binding properties and its ability to mediate ADCC against human tumor cells.

We have also investigated whether human T lymphocytes are able to distinguish the determinants of the point-mutated ras p21 proteins from normal ras p21. Cellular immunity to three synthetic peptides representing amino acids 5-17 of mutated ras p21 proteins with an exchange of normal glycine (G12) at position 12 by valine (V12), cysteine (C12) or aspartic acid (D12) was studied. T cell lines specific for peptides V12, C12, and D12 were able to be established from normal peripheral blood lymphocytes. The specificity of the T cell lines were assayed by T cell proliferation and production of cytokines. Cytotoxicity was demonstrated using as targets autologous EBV transformed B cells, pulsed with specific mutated ras peptides, and autologous EBV transformed B cells transfected with a vector carrying ras p21 protein constructs. T cells were MHC class II restricted. The results demonstrated that a human T cell specific immune response to point-mutated ras p21 proteins bearing a single amino acid substitution can be elicited from PBL, suggesting a potential for specific immunotherapy of human cancers.

Major Findings.

A monoclonal antibody (MAb), designated D612 (IgG2a), has been shown to react with a 48 kDa glycoprotein. An immunohistochemical survey of the tissue distribution of the D612 antigen showed that 70-80% of the primary or metastatic colorectal carcinomas were positive, as well as normal epithelium of the small and large intestine and the stomach. It was not found in normal specimens covering a wide range of non gastrointestinal tissues. D612 was found to mediate antibody-dependent cellular cytotoxicity (ADCC) in conjunction with normal human PMNC against antigen-positive colon tumor cell lines. Exposure of PMNC to IL-2 resulted in a 2- to 3-fold increase in specific ADCC activity.

Interleukin-6 (IL-6) can greatly increase the lytic activity of human LAK cells and can augment human NK cell activity. We have investigated the effects of human rIL-6 (hrIL-6) on ADCC activity of human PMNC using three distinct anti-colorectal carcinoma MAbs, D612, 17-1A and 31.1, to mediate ADCC activity. An increase in ADCC activity was observed for all three MAbs after PMNC were incubated with hrIL-6. The optimal ADCC activity was observed at a concentration of 100 U/ml of hrIL-6. hrIL-6 treatment did not augment non-specific (non MAb mediated) cytotoxicity. ADCC activity was increased further when PMNC were costimulated with 100 U/ml of hrIL-2 and 100 U/ml of hrIL-6. This suggests a potential synergistic effect in the use of the combination of hrIL-2 and hrIL-6 on ADCC activity in human PMNC.

We have investigated the effects of the introduction of the hrIL-6 gene on the expression of certain antigens of the human colorectal carcinoma cell line, HT-29. The human rIL-6 gene was inserted in a retroviral expression vector, pLNCXII, and was introduced into HT-29 cells by lipofection; G418 selected cells were cloned and screened for IL-6 production. IL-6 secreting clones were further analyzed by functional assay using the B9 cell bioassay and a clone designated HT-29pIL-6 was established and shown to be producing high levels of IL-6 (960 pg/ml/10<sup>6</sup> cells/24h). Little or no change in growth rates between HT-29pIL-6 and parental HT-29 cells were observed as determined by cell counts. Flow cytometric analysis was undertaken to investigate whether the transfection of the IL-6 gene in HT-29 cells could alter the expression of cell surface antigens. The level of CEA, HLA class I and class II and ICAM-1 expression was measured on the surface of untreated HT-29 cells, HT-29 cells treated exogenously with IL-6, HT-29pIL-6 and HT-29 cells transfected with an irrelevant gene, a human Ig  $\kappa$  chain. The results suggest that the change in the expression of CEA in HT-29 cells upon introduction of the IL-6 gene is selective. The percent of HT-29 cells expressing HLA class I and HLA class II antigens remains unaltered by the introduction of IL-6 into the cells. The expression of ICAM-1 was shown to decrease slightly.

The possibility of creating a T cell capable of secreting anti-tumor Ig was investigated by introducing MAb D612 heavy chain (HC) and light chain (LC) genes sequentially into MOLT-4 cells. Phenotypic analysis of the transfected MOLT-4 cells indicated that the level of expression of most lymphocyte markers (CD3, CD4, CD5, CD7, CD8, HLA class I and HLA class II) was similar for both the transduced clone and the parental MOLT-4 cells. Ig secreted by one clone (MTcD612) was purified and separated by SDS-PAGE. The migration of HC and LC was similar to the parental MAb D612. Western blot analysis using antihuman HC and LC antibody confirmed that the Ig secreted by MTcD612 was chimeric in nature. Inhibition assays showed that chimeric D612 secreted by transduced cells was able to completely block the binding of the <sup>125</sup>I-labeled parental D612 MAb. The results indicated that the reactivity of the chimeric MAb was similar to the parental D612 MAb. Lytic activity of human tumor cells mediated by chimeric D612 was at least as efficient than that mediated by the native D612 MAb. This is the first report of the secretion of any functional chimeric IgG by T cells and suggests a potential role of TILS transduced with anti-cancer antibody genes in cancer therapy.

The effects of recombinant human macrophage colony-stimulating factor (hrM-CSF) on the ADCC activity of human monocytes were investigated. Human peripheral blood monocytes were preincubated with hrM-CSF at various concentrations and then used as effector cells in an <sup>111</sup>In release assay. Cell lines LS-174T, CBS (human colorectal carcinomas) and KLE-B (poorly

differentiated human endometrial carcinoma) were used as targets to react with anti-colorectal carcinoma MAbs D612, native CC49 and chimeric CC49. Optimal lytic activity was observed at a concentration of 100 U/ml of rhM-CSF. A significant augmentation in ADCC activity could be obtained by treatment of monocytes with rhM-CSF for 3 days. Monocytes from seven donors were tested for their ability to respond in ADCC after pretreatment with rhM-CSF and activity was enhanced in all seven. However, the degree of augmentation varied among different donors as did the base line ADCC activity in the absence of rhM-CSF. These results indicate that rhM-CSF can augment ADCC of human peripheral blood monocytes using MAbs to human carcinoma suggesting a potential role for rhM-CSF in cancer immunotherapy.

We investigated whether human T lymphocytes are able to distinguish the determinants created by mutated ras proteins from the normal ras protein. Cellular immunity to three synthetic peptides representing amino acids 5-17 of mutated ras p21 proteins with an exchange of normal glycine (G12) at position 12 by valine (V12), cysteine (C12) or aspartic acid (D12) was studied. T cell lines specific for peptides V12, C12, and D12 were established from normal peripheral blood lymphocytes (PBLs) using four discontinuous stimulation cycles *in vitro* with single point-mutated peptides. The specificity of the T cell lines was assayed by T cell proliferation, production of cytokines upon stimulation with specific peptides, and cytotoxicity to autologous <sup>111</sup>In-labeled target cells. Cytotoxicity assays were performed by using (a) autologous EBV transformed B cells pulsed with specific mutated ras peptides, and (b) autologous EBV transformed B cells transfected with vector PRV3 (G12), PRV4 (V12), PRV10 (C12) and p1529 (neo control). Phenotypes of the peptide-specific T cell lines were all CD3+, CD4+ and CD8- cells. Induction of proliferation as well as interferon- $\gamma$ , IL-2 and IL-6 but not IL-4 secretion was observed when the corresponding peptide was used for stimulation. Specific cytotoxic T cell activity was detected when the corresponding mutated ras p21 peptide was used to pulse the target cells, or when the target cells used were transfected with the vector carrying the corresponding mutated ras protein. The peptide specific T cells were MHC class II restricted. The results demonstrated that a T cell specific immune response to mutated ras p21 protein bearing a single amino acid substitution could be elicited from human PBL, suggesting a potential for specific immunotherapy of human cancers.

### Publications

Tsang KY, Kashmiri SVS, DeFilippi R, Qi CF, Calvo B, Shu L, Nieroda CA, Greiner JW, Schlom J. A human T cell line engineered to secrete chimeric monoclonal antibody. *J Immunother* 1993;13:143-152.

Tsang KY, Kashmiri SVS, Qi CF, Nieroda C, Calvo B, De Filippi R, Greiner JW, Primus FJ, Schlom J. Transfer of the IL-6 gene into a human colorectal carcinoma cell line and consequent enhancement of tumor antigen expression. *Immunol Letters* In Press.

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

PROJECT NUMBER  
 ZO1 CB 09028-03 LTIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Recombinant Vaccines for Active Specific Immunotherapy of Human Carcinoma

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

<b>PI:</b> Judy Kantor	Expert	LTIB, DCBDC, NCI
<b>Others:</b> Chrysanthi Paranavitana	Biotech Fellow	LTIB, DCBDC, NCI
Jinji Akagi	Visiting Fellow	LTIB, DCBDC, NCI
Scott Abrams	Senior Staff Fellow	LTIB, DCBDC, NCI
Roberto Bei	Visiting Fellow	LTIB, DCBDC, NCI

Continued on page 2

COOPERATING UNITS (if any)

P. Snoy, FDA; S. Creekmore, BRMP, DCT, NCI; J. Greenblatt, CTEP, DCT, NCI; H. Kaufman, Boston University Medical School, Boston, MA; M. Hamilton, COP, DCT, NCI.

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 10.7

PROFESSIONAL: 8.1

OTHER: 2.6

CHECK APPROPRIATE BOX(ES)

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

A

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

Certain tumor associated antigens (TAAs) represent potential targets for active specific immunotherapy. Human carcinoembryonic antigen (CEA) is a 180 Kd glycoprotein which is overexpressed in human colorectal, gastric, pancreatic, breast and non-small cell carcinomas. CEA is an oncofetal protein and is considered to be weakly immunogenic in humans. Humoral or cell mediated responses to CEA have not been well documented in normal or cancer patients. The copresentation of CEA with a strong immunogen such as vaccinia virus would represent a logical approach to inducing anti-CEA responses for tumor immunotherapy. We have constructed and characterized a recombinant vaccinia virus expressing human CEA and have used it as an immunogen to study its effect on tumor growth in mice bearing CEA-expressing tumors. Rodent tumors do not express CEA. In order to develop a model system for active anti-CEA therapies, we have transduced a mouse colon adenocarcinoma cell line, MC-38, with human CEA. These tumors grow in syngeneic C57BL/6 mice and will eventually kill the animal. We have used this tumor model to evaluate the efficacy of our recombinant vaccine to prevent tumor growth in mice and its ability to elicit cell mediated and humoral anti-CEA immune responses. Animals immunized with the recombinant vaccine were resistant to challenge with the syngeneic tumor cells expressing CEA. Moreover, when mice having a palpable CEA tumor burden were immunized with the recombinant vaccine tumor growth was greatly reduced or eliminated. The recombinant vaccine immunized animals developed anti-CEA antibody titers and demonstrated a strong DTH response to CEA-expressing tumor cells. T cells isolated from immunized mice responded specifically to soluble CEA and could also mediate lysis of the CEA-expressing tumor cell line. No toxicity was observed in these animals. Immunogenicity and safety of this recombinant vaccine was tested in non human primates. Animals immunized with the recombinant vaccine developed strong anti-CEA antibody responses and specific DTH responses. PBLs from immunized monkeys were found to proliferate in response to CEA stimulation. Blood counts and differentials and hepatic and renal chemistries remained normal in all animals throughout the study and for up to 1 year following the primary immunization.

**Professional Personnel continued:**

Sam Zaremba	Senior Staff Fellow	LTIB, DCBDC, NCI
Joan Karr	Biotech Fellow	LTIB, DCBDC, NCI
Diane Eggensperger	Senior Staff Fellow	LTIB, DCBDC, NCI
Mary Lou Cutler	Expert	LTIB, DCBDC, NCI
Laura Masuelli	Visiting Fellow	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

**MAJOR FINDINGS**

A 2.4 kb cDNA clone, containing the complete coding sequence of CEA was isolated from a human colon tumor cell library and inserted in the vaccinia virus transfer vector pSC-11. This plasmid was developed method for inserting and selecting foreign genes which have been inserted into the vaccinia virus genome. The expression of the CEA gene is under the control of an early vaccinia virus promoter p7.5. This plasmid also contains viral TK gene segments which will direct the insertion of the CEA gene into the nonessential TK gene of the parental wild type vaccinia virus, WR. A recombinant vaccinia virus expressing CEA was obtained by homologous recombination of the plasmid with the wild type vaccinia virus, WR. The expected genomic structure of the recombinant virus was confirmed by restriction endonuclease digestion and Southern hybridization. The parental strain of vaccinia virus and the recombinant DNAs were analyzed using the restriction endonuclease Hind III. The cellular localization of the CEA protein was determined by immunofluorescent staining with the CEA specific MAb COL-1. The cells infected with the recombinant vaccinia virus showed distinctive cell surface staining with the MAb COL-1 under fluorescence. The recombinant vaccinia virus was able to express CEA and was able to insert the molecule into the cellular membrane, consistent with the normal cellular localization. Animals immunized with this recombinant vaccinia virus developed anti-CEA antibody responses.

Rodent tumors do not express CEA. In order to test the efficacy of a CEA recombinant vaccinia virus vaccine, a rodent tumor model expressing CEA was developed. The MC-38 mouse colon adenocarcinoma cell line was transduced with a retroviral vector containing human CEA. Recombinant colonies were selected in G418, cloned and tested for their expression of CEA. Two clones which express high levels of CEA were extensively characterized. Immunofluorescence assays were performed to evaluate the level of cell surface expression. The level of CEA expression in these clones was considerably higher than that found in moderately differentiated human carcinoma cell line, WiDr and were comparable to those found on the human colon carcinoma cell lines GEO and CBS. These cell lines are among the highest CEA-expressing cells reported. Further analysis demonstrated that the CEA expressed in one of the transduced clones had a similar molecular weight to native CEA (180,000) while the other cell line expressed a single glycosylated 70,000 dalton immunoreactive product. Seven CEA-specific MAbs reacted with the protein products of both clones. The CEA gene present in the 70Kd clone was sequenced and found to contain a deletion in two of the three repeated domains present in CEA. Both clones formed tumors when transplanted by subcutaneous injection in C57BL/6 mice. These tumors grew at approximately the same rate as the MC-38 untransduced line and the tumors from both cell lines eventually killed the mice. These cell lines were used as reagents in a active immunotherapy model using CEA as a target.

Anti-tumor activity and specific immune responses were induced in C57BL/6 mice after immunization with a WR strain recombinant vaccinia virus expressing CEA. The WR strain of vaccinia virus is a rodent strain whose virulence in mice is minimal. In order to develop a recombinant CEA vaccine that would be potentially safe and effective for the treatment of

patients whose tumors express CEA a different strain of vaccinia virus would have to be used. To this end we constructed a recombinant virus expressing CEA in the New York City strain of vaccinia virus (rV-CEA).

Studies were conducted to determine if rV-CEA in the New York City strain of vaccinia virus could prevent the establishment of tumor transplants. Two types of controls were utilized in this tumor model. First, the MC-38 colon carcinoma cells with CEA(+) and without CEA(-) the transduced CEA gene were used to determine if anti-tumor effects were specifically directed against CEA. Second, wild type, V-NYC vaccinia virus was used as an immunogen to determine if the immune responses generated were the consequence of the inserted human CEA gene. Three immunizations of  $1 \times 10^7$  pfu were given fourteen days apart. Seven days after the last immunization  $2 \times 10^5$  tumor cells were transplanted by subcutaneous inoculation. Neither V-NYC nor rV-CEA as immunogen conferred any protection against growth of transplanted CEA(-) tumors. The tumors from all 10 mice in each group grew rapidly at the same rate. CEA(+) and CEA(-) tumors grew at the same rate in animals receiving no virus immunizations and at the same rate in mice who had received V-NYC immunizations. Mice immunized with rV-CEA inhibited the growth of the CEA(+) tumors but failed to inhibit the growth of the CEA(-) tumors. These animals remained tumor-free for 120 days. These animals were rechallenged with  $1 \times 10^6$  CEA(+) tumor cells and remained tumor free throughout an additional 120 day observation period. No toxic effects due to the administration of the rV-CEA or V-NYC were observed.

Studies were also conducted to determine if the immunization of mice with rV-CEA could inhibit the growth of an established tumor burden. Tumors were transplanted 7 days prior to the first immunization. Three immunizations were given 14 days apart. The growth rate of the CEA(-) tumors were the same in mice whether they had been immunized with rV-CEA or V-NYC. Similar growth rates were seen in the mice bearing CEA(+) tumors but immunized with V-NYC. In contrast, greatly reduced tumor growth was observed in all mice bearing CEA(+) tumors after immunization with rV-CEA. Three animals failed to develop tumors in this group and were rechallenged with CEA(+) tumors. These animals remained tumor free for another 120 day observation period. CEA(-) tumors, transplanted on the contralateral side grew at the site. No toxic effects due to the administration of rV-CEA were observed.

Anti-CEA antibodies were detected in all rV-CEA immunized mice. The dynamics of the increase in antibody titer were also monitored. After the first immunization there was a modest rise in anti-CEA titers which greatly increased after the second and third immunizations.

Cell mediated immune responses to rV-CEA were also studied. DTH reactions in mice immunized with rV-CEA or V-NYC were measured by footpad swelling after challenge with X-irradiated tumor cells. Seven days after the last immunization, one footpad was given CEA(+) irradiated cells and the other footpad was given CEA(-) irradiated cells. Mice immunized with PBS or V-NYC had no or minimal footpad swelling 48 hours after injection of the tumor cells. However, a majority of the mice (14/20) immunized three times with rV-CEA demonstrated a DTH reactivity to the CEA(+) tumor cell line. It was also observed by positive DTH responses that three rV-CEA immunizations were better than two immunizations. Splenic T cells isolated from immunized and non immunized mice 28 days after the third and final vaccinia challenge were examined for functional competence and antigen specificity, indicated by proliferation in response to various stimuli. Of the three groups of T lymphocytes tested only those from mice immunized with rV-CEA were able to respond to soluble CEA. Splenic T lymphocytes isolated directly from rV-CEA and V-NYC immunized mice were also evaluated in vitro for cytotoxicity. Lymphocytes taken five days after a second vaccinia challenge from animals immunized with rV-CEA but not from animals immunized with V-NYC were able to mediate lysis of the CEA(+) tumor cell line.

The safety and immunogenicity of this recombinant vaccine were evaluated in rhesus monkeys. Included in the CEA family of genes is NCA or normal cross-reacting antigen. NCA expression appears on the surface of human granulocytes and to some extent on rhesus monkey

granulocytes. We were concerned that if we induced immune responses to CEA with vaccinia we could also induce responses to CEA related antigens such as NCA. The rhesus monkey allowed us to address this issue.

Eight monkeys were immunized either three or four times by skin scarification with the recombinant vaccinia virus. Four monkeys were immunized with wild type vaccinia virus as a control. Sera from each of the monkeys were analyzed by ELISA for immunoreactivity to CEA, NCA and ovalbumin. Preimmune sera was negative to all three antigens. Animals immunized with the rV-CEA had titers greater than 1:1000 that were detected seven days following the second boost. Four animals had titers of greater than 1:5800. One monkey appeared to develop a titer to NCA of 1:1250, however an identical titer to ovalbumin was also detected in this animal, indicating a potential nonspecific reactivity. rV-CEA induces a strong response to epitopes expressed on CEA with little or no response to NCA-specific epitopes. Biological activity of the immunoglobulins produced by the rV-CEA immunizations was analyzed by ADCC using human PBMC as effectors and the CEA(+) murine cell line as the target. Specific lysis of CEA(+) tumor cells was observed only in the monkeys receiving the rV-NYC immunization, while no lysis was observed using the CEA(-) cell line as targets. ADCC activity of the serum from rV-CEA immunized monkeys was enhanced using IL-2 activated human PBMC.

Monkeys were tested for DTH responses 7 days following their last immunization. All but one monkey responded with a positive DTH response to UV-inactivated vaccinia virus. None of the monkeys responded to the ovalbumin control antigen. Seven of the eight monkeys immunized with rV-CEA responded to purified CEA while in contrast none of the V-NYC monkeys had a positive DTH response to CEA. These results along with lymphoproliferative assay results demonstrated the ability of the rV-CEA vaccine to elicit cell mediated immune responses to CEA in monkeys.

Monkeys receiving V-NYC were compared to monkeys immunized with rV-CEA with respect to temperature, weight, regional lymphadenopathy and the presence of splenomegaly and hepatomegaly. Animals were tested for complete blood count, differential and hepatic and renal chemistries. Complete blood counts remained within the normal ranges throughout the study for both the wild type and recombinant immunized animals. Differential blood counts also remained normal in all animals throughout this study. Hepatic function was assessed by measuring serum albumin, bilirubin, SGOT, SGPT, and GGTP levels. No significant differences were found in these values between the control or recombinant immunized animals through out this study. Renal function was assessed by BUN and creatinine serum levels. There was no difference between the immunized animals and normals values throughout the study. This study also demonstrated that rhesus monkeys immunized with rV-CEA did not elicit any apparent immune responses against NCA epitopes.

## PUBLICATIONS

Kantor J, Irvine K, Abrams S, Kaufman H, DiPietro J and Schlom J. Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia virus vaccine. *J Natl Cancer Inst* 1992, 84:1084-1091.

Kantor J, Irvine K, Abrams S, Snoy P, Olsen R, Greiner J, Kaufman H, Eggensperger D, and Schlom J. Immunogenicity and safety of a recombinant vaccinia virus expressing the carcinoembryonic antigen gene in a nonhuman primate. *Cancer Res* 1992, 52:6917-6925.

Publications In Press

Irvine K and Schlom J. Induction of delayed type hypersensitivity responses by monoclonal anti-idiotypic antibodies to tumor cells expressing carcinoembryonic antigen (CEA) and tumor associated glycoprotein (TAG-72). *Cancer Immunol Immunother* (In Press).

Irvine K, Kantor J, and Schlom J. Comparison of a CEA-recombinant vaccinia virus, purified CEA, and an anti-idiotypic antibody bearing the image of a CEA epitope in the treatment and prevention of CEA-expressing tumors. *Vaccine Res* (In Press).



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

PROJECT NUMBER  
 ZO1 CB 09029-01 LTIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Host Immune Responses to Human Carcinoma Antigens Induced by Recombinant Vaccines

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

<b>PI:</b> Scott Abrams	Senior Staff Fellow	LTIB, DCBDC, NCI
<b>Others:</b> Mark Dobrzanski	Biotech Fellow	LTIB, DCBDC, NCI
Judy Kantor	Expert	LTIB, DCBDC, NCI
S. Zaremba	Senior Staff Fellow	LTIB, DCBDC, NCI
Yoomie Chung	Senior Staff Fellow	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)

Dr. J. Hamilton, NMOB, NCI; Drs. H. Kaufman, D. McAneny, Department of Surgery, Boston University Medical Center, Boston, MA; Dr. K. Lyerly, Department of Surgery, Duke University Medical Center, Durham, NC

LAB/BRANCH Laboratory of Tumor Immunology and Biology

SECTION Experimental Oncology Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 4.6	PROFESSIONAL: 3.2	OTHER: 1.4
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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.)

Our laboratory is investigating several well-characterized human carcinoma antigens (Ag), including carcinoembryonic antigen (CEA) and point-mutated ras p21. The over-expression of tumor-associated Ag and/or the neo-expression of tumor-specific epitopes may represent selective or unique targets for immune recognition, particularly by T lymphocytes which have been implicated as integral effector elements for host antitumor activity. Thus, we have begun to evaluate the repertoire of host cellular immune responses induced by active immunization to CEA and point-mutated ras p21. In a murine C57BL/6 (H-2b) model, we have examined whether active immunization with a recombinant vaccinia virus expressing human CEA (rV-CEA) could induce T cell responses, which might correlate with and/or participate in the tumor rejection mechanism. Overall, we have shown that: (1) T cells from rV-CEA mice elicited Ag-specific proliferation to soluble CEA protein; (2) T cells from rV-CEA mice mediated specific lysis of CEA+ bearing tumor target cells; and (3) splenocytes from rV-CEA mice expressed antitumor activity through adoptive immunotherapy. Similarly, we have shown that lymphocytes from rV-CEA immunized rhesus monkeys displayed Ag-specific proliferation responses. More recently, we have examined whether active immunization with short synthetic peptides, which mimic point-mutated epitopes of ras p21 proteins, could induce T cell responses in a murine BALB/c (H-2d) model. Overall, we have shown that: (1) mice immunized with a 13-mer ras peptide, containing the substitution of glycine at position 12 for valine, demonstrated specific T cell proliferation to the immunizing peptide. No autoimmune response was detectable to the normal ras p21 sequence; (2) CD4+ T cells (line and clones) were established *in vitro*, which retained peptide specificity; and (3) CD4+ T cells secreted a spectrum of cytokines (e.g., IL2, IFN- $\gamma$ , TNF or GM-CSF) and some effectors expressed cytotoxicity against tumor target cells incubated with the specific peptide. Taken collectively, we demonstrate that a rV-CEA construct and point-mutated ras p21 peptides are immunogenic in animal models, which may have important implications for active specific immunotherapy of human carcinoma.

## Major Findings

CEA is a 180-kd glycoprotein over-expressed in a variety of human carcinoma cancers, including: colorectal, gastric, pancreatic, breast and non-small cell lung. The fact that CEA is highly expressed on a diversity of human carcinomas suggests that it may be useful as a selective target Ag for immune intervention. However, there is virtually nothing known about the immunogenicity of CEA in humans. One possibility is that CEA may lack sufficient immunogenicity for the induction of effective host immune responses. One potential approach to circumvent weak immunogenicity involves the use of recombinant virus vectors, such as vaccinia virus, engineered to express newly introduced genes. Recombinant virus vectors potentiate immunogenicity presumably by modifying the normal mechanism and pathway for extrinsic Ag processing and presentation, leading to a more antigenic form and the production of a milieu of cytokines resulting from enhanced immune interactions. Here, cellular immune responses to CEA were examined in vitro for proliferation and cytotoxicity.

Splenic T cells isolated from unimmunized or immunized mice 28 days after their third and final vaccine challenge were examined for functional competence and Ag specificity by proliferation to various stimuli. Only T cells from mice immunized with rV-CEA responded by proliferation to soluble CEA. Ag specificity to CEA was revealed using ovalbumin, an irrelevant soluble Ag, which failed to stimulate T cells from these mice. T cells from rV-CEA mice also responded to ultraviolet (UV)-inactivated wild type vaccinia virus as antigenic stimulation. Furthermore, T cells from mice receiving wild type vaccinia virus (V-NYC), but not from unimmunized mice, demonstrated reactivity to UV-inactivated vaccinia virus, thus confirming specificity to this viral Ag and functional competence of the V-NYC group. All three groups of lymphocytes responded strongly to Con A as a general measure of functional T cell competence.

In addition to splenic T cells, we examined proliferation responses from T cells derived from the inguinal and periaortic lymph nodes draining the site of immunization. Lymph node T cells from rV-CEA mice, as with splenic T cells, responded by proliferation to CEA, UV-inactivated vaccinia virus and Con A, but not to ovalbumin. In contrast, lymph node T cells from V-NYC mice responded by proliferation to UV-inactivated vaccinia virus and Con A, but not to CEA or ovalbumin. Thus, immunization with rV-CEA, but not with V-NYC, induced systemic, peripheral T cell responsiveness to CEA, which correlated with an efficient antitumor effect in vivo.

Splenic T cells, isolated directly from rV-CEA or V-NYC immunized mice, were also evaluated in vitro for cytotoxicity. T cells were obtained from mice 5 days after a boost, since we reasoned that primary cytotoxic T cell activity would be maximal shortly after a secondary challenge. We showed that T cells from mice immunized with rV-CEA, but not with V-NYC, mediated lysis of CEA<sup>+</sup>-transfected murine tumor cells. In contrast, under similar incubation conditions with both effector groups, only background levels of lytic activity were detectable against the parental CEA<sup>-</sup> tumor target. In the presence of Con A, which circumvents Ag-specific recognition and facilitates lectin-dependent cellular cytotoxicity, both effector groups efficiently lysed the CEA<sup>-</sup> target cells, indicating that T cells from mice receiving V-NYC, indeed, were lytically active and that this tumor cell line was not intrinsically resistant to lysis.

Our findings support the induction of cell-mediated immune responses as a consequence of rV-CEA challenge. CEA-specific T cell proliferation and cytotoxicity were inducible and correlated with antitumor effects in vivo. To determine whether cell-mediated immunity is important for tumor rejection, however, we have initiated cellular adoptive immunotherapy experiments. To that end, fresh splenocytes from mice immunized with rV-CEA were passively transferred to syngeneic naive tumor-bearing recipients, and localized tumor growth measured over time. Normal splenocytes or splenocytes from V-NYC mice served as effector cell specificity controls as well as controls for determination of any specific antitumor activity. Recipient mice received sublethal (500 R) g-irradiation prior to tumor transplant, which has been reported to abrogate host-dependent immune responses and to maximize the impact of the adoptively transferred effector cells. Donor splenocytes were removed from mice 3-4 weeks after their third immunization, which

coincided with their transfer ( $> 60 \times 10^6$ /mouse, i.v.) to 3-day, tumor-bearing recipients. Our preliminary findings revealed that splenocytes from rV-CEA mice delayed the onset of tumor growth and prolonged host survival, as compared to the controls. These findings support the hypothesis for immunologic involvement and establish the basis and rationale in future work to define the relevant effector cell(s).

The safety and immunogenicity of rV-CEA was also explored in a non-human primate model. Peripheral blood mononuclear cells (PBMC) were isolated from rhesus monkeys 6 or 12 months following their final immunization with either rV-CEA or V-NYC. As with the murine studies, we demonstrated that PBMC from rV-CEA monkeys, but not from V-NYC monkeys, responded by proliferation to soluble CEA. Reactivity to ovalbumin, however, was comparable to that of the control cultures without any Ag, confirming the specificity of CEA stimulation. Both groups of immunized monkeys responded equally well by proliferation to UV-inactivated vaccinia virus, revealing effective priming to the vector, and to Con A, as a general measure of T cell competence. Collectively, such proliferation patterns were observed in three representative monkeys from each vaccinated group. Interestingly, the observations for CEA-specific proliferation at 6 or 12 months implicate the induction of immunologic memory with potential "long term" immunity.

A few reports now indicate that point-mutated forms of ras p21, administered as peptides which reflect those alterations, are immunogenic in murine and human models. Functional characterization of the responding T cell populations, however, remains to be elucidated, which is important for understanding potential effector mechanisms required for antitumor activity. To that end, peptide 13-mers were synthesized corresponding to the altered and normal regions of the ras p21 proteins. In a murine BALB/c (H-2<sup>d</sup>) model, we evaluated the immunogenicity of these ras peptides and the functional characteristics of the resulting T cell responses. We selected the BALB/c (H-2<sup>d</sup>) mouse because the proposed tumor model will involve cells of the same genetic background.

Our initial studies have focussed on the glycine (G) to valine (V) substitution at position 12, which is found in many human carcinomas. Using a strategy similar to that described by Peace et al. (J. Immunol. 146:2059, 1991), ras peptides were synthesized representing position 5-17 of the normal and aberrant forms of the ras p21 proteins. We demonstrated that BALB/c mice immunized in adjuvant with a ras peptide containing the V12 point mutation mounted a productive immune response, as measured by proliferation of in vivo-primed T lymphocytes. In contrast, no significant immune response was detected under any experimental condition with the normal peptide (G12), indicating the absence of autoimmune reactivity. A T cell line and clones were established from appropriately immunized mice, which elicited potent, peptide-specific proliferation responses. Thus, in vivo immunization with this ras[V12] peptide correlated with the induction and expansion of Ag-specific T cell subsets.

Phenotypic analysis revealed the proliferating T cells as ab<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup>, with a cytokine profile consisting of IL2, IFN- $\gamma$ , TNF and/or GM-CSF, characteristic of the T<sub>H</sub>1 subsets. Such a set of cytokines tend to associate with cell-mediated immunity. Moreover, we demonstrated that the T cell line and some clones displayed Ag-specific lysis against Ia<sup>D+</sup> B cell lymphoma target cells incubated exogenously with the V12 peptide. It is unknown whether such peptide-induced T cell clones can recognize and attack tumors bearing endogenous Ag, although we plan to explore those possibilities using syngeneic fibroblasts transfected with the appropriate point-mutated ras p21 gene. Nevertheless, the observations that the V12 peptide-specific T cells produced a spectrum of cytokines and certain subsets expressed lytic function, support the hypothesis for biologically relevant immune interactions. Preliminary experiments indicated that C12 (cysteine) and R12 (arginine) peptides were immunogenic, as measured by proliferation of in vivo-primed T lymphocytes.

In our preliminary experiments, a role for CD8<sup>+</sup> T cells was much less evident, which may reflect, in part, the methodology utilized for in vivo immunization and/or in vitro propagation. The inability to effectively propagate such CD8<sup>+</sup> T cells may be due to inadequate antigenic stimulation, as investigations in other systems report optimal induction and expansion of cytotoxic T cells with peptide epitopes of 8-10

(commonly, 9) residues and/or displaying unique major histocompatibility complex class I binding motifs. Such possibilities are currently being explored in this model.

An important goal of this work for both CEA and point-mutated ras p21 is to define and characterize the most relevant effector mechanisms induced by active immunization for maximal antitumor responses. Accordingly, we plan to explore in each system multiple approaches, involving combinations of recombinant vaccines, immunogenic peptides and/or cytokines. We plan to enter phase I clinical trials with rV-CEA as a prototypic vaccine for active specific immunotherapy of human carcinoma.

#### Publications

Kantor J, Irvine K, Abrams S, Kaufman H, DiPietro J, and Schlom J. Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia virus vaccine. *J Natl Cancer Inst* 1992;84:1084-1091.

Kantor J, Irvine K, Abrams S, Snoy P, Olsen R, Greiner J, Kaufman H, Eggensberger D, and Schlom J. Immunogenicity and safety of a recombinant vaccinia virus vaccine expressing the carcinoembryonic antigen gene in a nonhuman primate. *Cancer Res* 1992;52:6917-6925.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CB 09022-07 LTIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Cytoskeletal Proteins in Oncogenes Transformation and Human Neoplasia

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

PI:	Herbert Cooper	Chief, Cell & Molec. Physiol Section	LTIB, DCBDC, NCI
Others:	G.L. Prasad	Visiting Scientist	LTIB, DCBDC, NCI
	Rebecca Fuldner	Senior Staff Fellow	LTIB, DCBDC, NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Cell and Molecular Physiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF 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.)

We have continued our studies on the relationship of suppression of tropomyosin (TM) synthesis to neoplastic transformation. Previous observations have led us to hypothesize that TM suppression is a causal event in neoplastic transformation. We have obtained evidence supporting this hypothesis by restoring expression of TM1, one of two suppressed tropomyosins, in the v-Ki-ras-transformed NIH3T3 cell line. DT, by retroviral mediated cDNA transfer. Cell clones expressing the cDNA had elevated levels of TM1 and lost the ability to grow under anchorage-independent conditions. They also did not participate in formation of tumors in athymic mice. However morphological reversion was incomplete. Elevated levels of TM1 synthesized in the transduced clones were only partially utilized in the cytoskeleton and disrupted microfilament bundles were only partially restored. Abnormal levels of TM1 homodimers and of crosslinked homodimers were produced which may associate poorly with the cytoskeleton. Studies with double insertion of both TM1 and TM2 suggest that under these conditions complete reversion of the transformed phenotype may occur.

### Major Findings

We have continued our studies on the relationship of suppression of tropomyosin (TM) synthesis to neoplastic transformation. Previous work has established that expression of two higher  $M_r$  TMs (TM1 and TM2) was consistently reduced or eliminated in murine cells transformed by a variety of retroviral oncogenes, DNA tumor viruses, chemical carcinogens, or transforming growth factors (TGF- $\alpha$ ). We have also examined 6 commonly studied cell lines derived from human breast carcinomas and found them all to be defective in expression of TM1, together with other abnormalities of TM expression. This suggests that tropomyosin suppression may be involved in human carcinoma. The inference that TM suppression played a causal role in neoplastic transformation was supported by the observation that restoration of TM1 expression to above normal levels in ras transformed NIH3T3 cells (DT cells) by retroviral-mediated cDNA insertion (producing DT/S clones) resulted in loss of anchorage-independent growth capability and delayed onset of tumor outgrowth in athymic mice. When tumors did arise, they no longer expressed the inserted TM1 cDNA, indicating that cells expressing elevated amounts of TM1 did not participate in tumor formation. Evidently some aspect of *in vivo* growth suppressed expression of the cDNA insert.

Our recent efforts have been directed at further understanding of the suppression of the transformed phenotype in response to elevation of TM expression. The following questions have been considered:

- I. It has been proposed that the role of TM in the nonmuscle cell includes stabilization of actin microfilaments, which are known to be severely disrupted in transformed cells. We examined the appearance of microfilament bundles by fluorescence microscopy and found that transformed cells expressing elevated levels of TM1 (DT/S clones) exhibited a partial restoration of microfilament bundle formation which coincided with increased cytoplasmic spreading ('flattening'). This supports the notion that the mechanism of microfilament disruption by oncogenic agents includes destabilization due to TM deficiency. If microfilament disruption is a causal factor in neoplastic transformation, as has been proposed by others, then the mechanism of suppression of the neoplastic phenotype by elevation of TM expression would appear to be mediated by its ability to restore microfilament integrity. As outlined below, it is proposed to test this conclusion by site-directed mutagenesis experiments.
- II. With the exception of anchorage-independent growth, which was completely suppressed, the effects of elevation of TM1 expression on other aspects of the transformed phenotype were all partial (it is not known whether tumor growth would have been completely suppressed if TM1 expression had not been extinguished *in vivo*.) The basis for the lack of complete reversion was investigated. Since the locus of TM function, as far as is known, is in association with the actin microfilament, the cytoskeletal utilization of TM derived from cDNA was examined. This study showed that:
  - A. In unmodified transformed cells (DT cells), while TM1 synthesis was reduced to about 50% of normal cell levels, the utilization of available TM1 was virtually nonexistent. This agrees with earlier work showing that the utilization of TM may be specifically inhibited by transforming modalities.
  - B. In DT/S clone cells, although TM1 synthesis was at least 5 times normal levels, TM1 utilization was restored to only 50% of normal cell levels. Thus, a block to the utilization of TM continues, which can only be partially overcome by excess TM1 production. This may account for the finding of only partial restoration of microfilament integrity and incomplete reversion of the transformed phenotype.
- III. TM occurs *in vivo* as a coiled-coil dimer, and studies with muscle TMs indicate that the thermodynamically preferred dimer is a heterodimer between TM1 and TM2, as opposed to the homodimer forms, TM1:TM1 and TM2:TM2. This preference has not previously been verified for

fibroblast TMs. Moreover, it has been reported that heterodimers associate more stably than homodimers with actin microfilaments. In DT/S clones, TM1 is greatly overexpressed, but TM2, which was strongly suppressed in the parental DT cells by oncogene action, continues to be virtually absent. The dimer form in which TM1 was expressed and utilized in normal fibroblasts and in DT/S clones was studied to determine whether unusual dimer forms occurred. This study revealed that:

- A. In normal fibroblasts, TM1 homodimers are initially formed, which are gradually converted to heterodimers by a process of chain exchange. Thus, heterodimers are the stable form of TM *in vivo*, as previously reported for muscle cells. This finding documents the occurrence *in vivo* of the process of chain exchange, which had previously been described only in experiments *in vitro*.
- B. In both normal and transformed cells overexpressing TM1, large numbers of TM1 homodimers are formed which initially enter the cytoskeleton. With time, these are converted to heterodimers in normal cells. In transformed cells, TM2 is unavailable for chain exchange, so TM1 homodimers persist.
- C. In both normal and transformed cells overexpressing TM1 a previously undescribed TM1 homodimeric form occurs in significant amounts. In this form, the two TM1 moieties are covalently linked by a disulfide bridge between the single cysteine present in each molecule. This form apparently occurs to a small extent in normal cells, but in cells overexpressing TM1, large amounts are produced. Because of the crosslink, these TM1 homodimers are not susceptible to chain exchange and cannot be converted to heterodimers and they therefore form a stable component of the cytoskeleton. As a result, abnormally constituted actin microfilaments are produced by such cells. The function of such linked homodimers in either normal or TM overexpressing cells remains to be determined.

IV. As noted above, TM1 overexpression does not completely correct the defect in TM expression found in DT cells, because TM2 synthesis remains virtually absent. The poor cytoskeletal utilization of overexpressed TM1 in DT/S cells may arise because TM2 is unavailable to permit formation of the stable heterodimer form. Alternatively, some specific effect of *ras* expression may persist in inhibiting TM utilization. Moreover, the persistent deficiency of TM2 in DT/S cells may be the reason why only partial reversion of the transformed phenotype occurred. To study this question, DT/S cells, already overexpressing TM1, were further modified to express a cDNA for mouse TM2. Clones have been picked which now express both TM1 and TM2, in varying ratios, above the levels in either parental DT cells or in NIH3T3 cells. These clones are now being actively studied to determine the effects of this double modification on the transformed phenotype. To date we have observed a remarkable morphological reversion from the typical transformed growth pattern of DT cells to a completely flat revertant appearance in some of these clones. They are currently being tested for tumorigenicity. It is not known whether, and how quickly, expression of the pair of inserted cDNAs will be extinguished in the *in vivo* setting, as was the case for TM1 alone. Fluorescence microscopic examination of microfilament structure is in progress, as is examination of biochemical parameters, such as TM utilization and dimer formation.

V. Our previous findings indicated that TM1 overexpression in DT/S cells was extinguished *in vivo* after a short delay, following which tumor formation proceeded. This complicates an assessment of the true effect on tumorigenicity of TM replacement. Moreover, such extinction must be overcome if such constructs are to have any possible future therapeutic value. We are attempting to determine the basis for the extinction phenomenon.

- A. A new TM1 cDNA has been produced by PCR cloning which lacks 5' and 3' untranslated sequences (TMA). Since the original TM1 insert was derived from a human cDNA library, these regions differ from the endogenous TM1 transcript and may contain sequences which are susceptible to binding by down regulatory factors. TMA has been inserted into DT cells and clones have been derived. These are now under study to determine whether TM1 expression will continue to be extinguished *in vivo*.
- B. All of the above cDNA insertions were made using the cytomegalovirus (CMV) promoter to regulate expression of TM1 transcripts. It is possible that the CMV promoter is particularly vulnerable to extinction *in vivo*. To test this, reporter genes (CAT) are being cloned into the pBNC vector used to insert TM1. DT cells will be infected and selected for CAT expression under control of the CMV promoter. These will then be used to produce tumors in athymic mice and the tumors will be analyzed for persistence or extinction of CAT expression.
- C. Along the same lines, new vectors will be prepared in which TM1 and TM2 expression is controlled by other promoters, which may be more resistant to extinction. We are currently attempting to obtain an actin promoter for this purpose.

VI. All of the above work was done with the DT cell line of v-Ki-*ras* transformed NIH3T3 cells. The question arises whether the findings with *ras* are representative of other oncogenic modalities which would indicate that TM suppression played a causal role generally in neoplastic transformation. It has been shown that a variety of oncogenes and other transforming agents all cause suppression of TM expression. It remains to be seen whether TM restoration will cause reversion in these cases. We have therefore begun to transduce TM1 cDNA into cell lines transformed by other oncogenes. Currently we are deriving TM1 overexpressing clones from NIH3T3/*mos* and NIH3T3/*src* transformed lines. These are currently being examined for effects on anchorage independent growth and on other aspects of the transformed phenotype.

VII. All the above studies involve insertion of ectopic genetic sequences into malignant cells in order to suppress or reverse aspects of the neoplastic phenotype. Implicit in these studies is the possibility that these or related sequences may have ultimate value in a gene therapy approach to the treatment of human cancer. A necessary component of any such approach is the vector used for conveying the sequence into the target cell. The use of retroviral or other infectious vectors will be limited unless methods are found to target specific cell types. We have begun development of a model system for producing a retroviral vector that will be targeted specifically to cells of the colon epithelium. This involves generating a packaging cell line which will provide viral coats for the gene-carrying plasmid in which the MuLV *env* protein (GP70) which is responsible for host cell recognition is modified to redirect it to the colon cell as host. We are preparing constructs in which a portion of the GP70 protein involved in host cell recognition is replaced by a single chain antibody construct for the D612 antibody which was prepared by LTIB investigators. The D612 antibody, as described in earlier studies from LTIB, has high specificity for colon epithelial cells and recognizes a cell surface glycoprotein. The DNA sequence encoding the altered GP70 will be transfected into NIH3T3 cells by the pJ4 $\Omega$ EnvE vector, which we have obtained, and whose Env sequence is now being modified. Together with the pJ4 $\Omega$ gag-pol vector, the necessary functions will be provided to package appropriate gene-carrying vectors bearing the *psi* packaging signal. The system will be tested for elaboration of particles carrying reverse transcriptase and for its ability to carry reporter genes specifically into human colon cells and not into fibroblasts, lymphoid cells, etc.



## Future plans

Since the senior investigator anticipates retiring at the end of the current fiscal year, the following future plans are suggestions for the further progress of this project.

- I. Completion of studies on cell clones over-expressing both TM1 and TM2. Preliminary evidence suggests that double expressors may show complete suppression of the transformed phenotype. Effects of double TM expression on microfilament structure should be studied to determine whether transformation-induced microfilament disruption is completely reversed. This will provide strong evidence that TM suppression by oncogenic modalities is the basis for the microfilament disruption generally found, as well as providing further support for TM suppression as a causal step in transformation.
- II. Completion of studies to determine the basis for extinction of expression of ectopic TM cDNA under *in vivo* conditions.
  - A. Completion of studies with TMD cDNA to determine if extinction is dependent on regulatory signals in untranslated regions of the insert.
  - B. Completion of studies on the possible *in vivo* extinction of reporter genes under control of the CMV promoter to determine whether the extinction we have observed is specific for TM or is independent of the insert sequence but possibly related to the promoter employed.
  - C. Construction or acquisition of vectors which will allow transduction or transfection of TM cDNAs under control of other promoters which may be resistant to *in vivo* extinction. At present we are trying to obtain a vector containing the actin promoter. Through a combined effort by lawyers at the NCI Office of Technology Development and at the institution of the investigator who designed this vector, obtaining the vector has been delayed so far by 5 months. There is still no apparent end in sight to this obstruction.
- III. Completion of study of effects of TM1 replacement in cells transformed by *src* and *mos* oncogenes. This is necessary to determine whether our results to date are specific to the *ras* oncogene or may be more widely applicable.
- IV. Continuation of development of host-determining retroviral packaging line. This may demonstrate the feasibility of specifically altering viral recognition sequences for targeting of infectious vectors to specific host cells.
- V. Site-directed mutagenesis studies to examine the structural requirements for the TM molecule to antagonize the transformed phenotype.
  - A. TM is a highly  $\alpha$ -helical molecule with a characteristic heptad repeat structure which promotes the formation of elongated side-to-side coiled-coil dimers. These dimers interact with actin microfilaments to promote the stability which we theorize is responsible for antagonizing neoplastic growth. Certain features of TM structure are necessary for its proper configuration:
    1. The molecule can contain neither proline nor tryptophan, since these amino acids would break the ( $\alpha$ -helical structure).
    2. The proper sequence of hydrophobic and hydrophilic residues is required in the heptad repeat to promote coiled-coil dimerization.

3. The length of the molecule appears important, since shorter TM isoforms are not as effective as longer ones in associating with the actin microfilament
  4. The single *cys* residue of TM1 or TM2 permits the formation of disulfide bridges in TM homodimers. We have shown that such crosslinked dimers occur in large amounts in cells overexpressing TM1, particularly if TM2 is not available for chain exchange. Small amounts of this crosslinked form are also produced in normal cells. It is not known whether the occurrence of such crosslinked forms has any specific consequences for TM function.
    - B. It is proposed to produce a series of cDNAs altered by site-directed mutagenesis which will examine the importance of each of the above structural features in the ability of TM to antagonize the neoplastic phenotype.
      1. A series of cDNAs in which an amino acid at various locations is converted to proline. This should break the  $\alpha$ -helix at that point and interfere with dimerization and association with microfilaments.
      2. Forms with varying degrees of substitution to disrupt the heptad repeat structure. These should interfere more subtly with dimerization.
      3. Forms in which varying portions of the NH<sub>2</sub>- or COOH-termini or central regions are deleted.
      4. TM1 cDNA with a substitute for *cys*.
        - C. Each of these forms will be transfected into DT cells and expressing clones selected. These clones will be compared with DT/S clones for their loss of anchorage independent growth, for cytoskeletal utilization, for dimer formation, and for restoration of microfilament bundle formation.
- VI. Extension of TM studies to human neoplasia
- A. We have previously shown that all of 6 widely studied cell lines derived from human breast carcinoma lacked expression of TM1 (which is expressed by normal mammary epithelium) and also lacked expression of at least one other high M<sub>r</sub> TM. This is presumptive evidence that abnormalities of TM expression may be involved in human neoplasia. Further exploration of this area has been impeded by the lack of isoform-specific anti-TM antibodies. To date, we have not been able to produce a TM1 specific antibody, which would enable us to screen surgical tumor sections to determine if absence of TM1 expression is commonly found in breast cancer (available antibodies cross react extensively with other TM isoforms, which may not be suppressed).
    1. Attempt to produce TM1 specific antibody by producing large quantities of a fusion protein in bacteria which will express a TM1 specific peptide sequence coupled with a larger, cleavable carrier protein. This will be used to immunize rabbits which will be boosted with the cleaved TM1 specific peptide. The resulting antiserum will be affinity purified against bacterially produced TM1.
    2. Use of above antibody for immunohistochemical screening of surgical tissue specimens. Sequential sections will be stained with TM1-specific antibody and broad-specificity antibody. Loss of staining with TM1 specific antibody over regions of malignant cells will indicate specific reduction of TM1 expression. This approach should permit a determination of the frequency of TM1 suppression in breast cancer (and in other cancers), and the relationship of this characteristic to tumor progression, histological type, and prognosis.
  - B. Exploration of the possibility of reversion of neoplasia by TM insertion in mammary carcinoma cells.
    1. We have produced clones of MCF7 cells in which TM1 expression, originally completely suppressed, has been restored following retroviral mediated cDNA transfer. These clones await further study, which should include an assessment of anchorage-independent growth and tumorigenicity.

2. Since MCF7 cells have an extensive history in cell culture, results with them may not be representative, and studies should be performed on freshly derived breast cancer cell lines.

**Publications:**

Prasad, G. L., Valverius, E. M., McDuffie, E. and Cooper, H. L. cDNA cloning of a novel epithelial cell marker protein, HME1, that may be down-regulated in neoplastic mammary cells. *Cell Growth and Differentiation*. 3: 507-513, 1992.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CB 09003-11 LTIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

The Role of EGF-related Peptides in the Pathogenesis of Breast and Colon Cancer

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

Pi: David Salomon Chief, Tumor Growth Factor Section LTIB, DCBDC, NCI  
 Others: Nicola Normanno Visiting Fellow LTIB, DCBDC, NCI  
 Ralf Brandt Visiting Fellow LTIB, DCBDC, NCI  
 Nicholas Kenny Graduate Student LTIB, DCBDC, NCI

## COOPERATING UNITS (if any)

Dr. Robert Callahan, Chief, Oncogenetics. Section., LTIB, DCBDC, NCI; Dr. Marc Lippman, Director., Lombardi Cancer Center., Georgetown Univ., Washington, DC.; Dr. George Todaro, Bristol-Meyers Squibb, Seattle, WA.

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Tumor Growth Factor Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 4.0

PROFESSIONAL: 2.7

OTHER: 1.3

## CHECK APPROPRIATE BOX(ES)

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

B

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

Transforming growth factor  $\alpha$  (TGF $\alpha$ ), amphiregulin (AR) and cripto-1 (CR-1) are proteins that are structurally and in some cases functionally related to epidermal growth factor (EGF) in that TGF $\alpha$  and AR can bind to the EGF receptor (c-erb B). TGF $\alpha$  has been circumstantially implicated in the autocrine growth of a number of different human carcinoma cells such as breast and colon tumors. However, the regulation of expression of this growth factor and interference with its biological activity have not been thoroughly examined. Moreover, the relative levels of expression and biological function of AR and CR-1 in these malignancies are unknown. The present studies have demonstrated that MCF-10A human mammary epithelial cells are mitogenically responsive to exogenous EGF, TGF $\alpha$  or AR and that transformation of these cells with a point-mutated c-Ha-ras protooncogene but not with a c-erb B-2 protooncogene results in an increase in the expression of endogenous TGF $\alpha$ . Furthermore, overexpression of a human TGF $\alpha$  cDNA in these cells leads to their in vitro transformation. Addition of an anti-EGF receptor blocking antibody or an anti-TGF $\alpha$  neutralizing antibody can partially or completely inhibit the growth of the Ha-ras or TGF $\alpha$  transformed mammary cells suggesting that an external autocrine loop is operative in these cells. In contrast, AR expression is increased in both Ha-ras and c-erb B-2 transformed MCF-10A cells and the growth of these transformants can be inhibited by AR antisense phosphorothioate oligonucleotides demonstrating that AR is functioning as an autocrine intermediary in the transformation pathway that is utilized by both Ha-ras and erb B-2. Estrogens can increase the expression of TGF $\alpha$  mRNA and protein in estrogen-responsive human breast cancer cell lines such as MCF-7 or ZR-75-1 cells. Transient transfection assays in MCF-7 or ZR-75-1 cells using a plasmid containing the TGF $\alpha$  promoter ligated to either the chloramphenicol acetyltransferase (CAT) or luciferase genes have demonstrated that physiological concentrations of estrogens can induce a 5- to 50-fold increase in the activity of these reporter genes, suggesting that the TGF $\alpha$  promoter contains a cis-acting estrogen-responsive element(s) (ERE). MCF-7 or ZR-75-1 cells were infected with a recombinant amphotropic TGF $\alpha$  antisense mRNA expression vector. Expression of this antisense mRNA lead to a reduction in estrogen-induced TGF $\alpha$  protein production and to an equivalent degree of inhibition of estrogen-induced proliferation in these cells. Specific mRNA and immunoreactivity for AR and CR-1 have been detected in approximately 50% to 80% of primary and metastatic human colorectal tumors, whereas only 5% of normal adjacent colon or liver tissue express these genes. Likewise, immunoreactive AR and CR-1 was detected in approximately 70% of primary human breast tumors at a level that exceeded the level found in adjacent normal mammary epithelium.

## Major Findings

To determine if endogenous TGF $\alpha$  might be synthesized *in vivo* in the mammary gland and to ascertain if TGF $\alpha$  could be localized to a specific cell type(s) within the mammary gland, we have examined paraffin or frozen sections that were obtained from virgin, pregnant and lactating rat and human mammary tissues by *in situ* hybridization using a 35S-labeled TGF $\alpha$ -specific antisense RNA riboprobe to detect TGF $\alpha$  mRNA transcripts. Quantitation of the relative levels of TGF $\alpha$  mRNA in each tissue section was accomplished by assessing autoradiographic grain density in multiple areas of each section using a MCID computer-assisted digital image scanning processing system. A 35S-labeled TGF $\alpha$ -sense RNA riboprobe was utilized as a control to correct for nonspecific hybridization, which was generally 6- to 10-fold less than the hybridization intensity or grain density observed with the labeled TGF $\alpha$  antisense riboprobe. Expression of TGF $\alpha$  mRNA was observed in 80 to 90% of the ductal and alveolar epithelial cells in the virgin rat mammary gland while little hybridization was detected over the surrounding stroma. A qualitatively similar pattern of TGF $\alpha$  mRNA expression was observed in the epithelial cells in the pregnant rat mammary gland. However, during pregnancy there was an approximately 50% increase in the amount of TGF $\alpha$  mRNA which could be detected within the epithelial cell population. In addition, 10 to 15% of the adjacent but not distal stromal cells were also expressing elevated levels of TGF $\alpha$  mRNA. In the lactating rat mammary gland, there was a further 2- to 3-fold increase in the hybridization intensity in the epithelial cells using the TGF $\alpha$  antisense riboprobe as compared to grain density in the epithelial cells found within the virgin or pregnant rat mammary gland. Mammary tissue that was obtained from nulliparous and parous premenopausal women was also examined for TGF $\alpha$  mRNA expression. As in the rat mammary gland, TGF $\alpha$  mRNA expression could be localized to the epithelial cells of both the ducts and lobules. The level of TGF $\alpha$  mRNA expression increased approximately 2-fold in the epithelial cells during midpregnancy. These results suggest that TGF $\alpha$  may function as an autocrine growth factor *in vivo* for both ductal and alveolar mammary epithelial cells and that the increase observed during midpregnancy and lactation would suggest that certain mammotrophic hormones which are known to be elevated in the circulation during these periods might be involved in regulating the expression of this growth factor *in vivo*. The elevation of TGF $\alpha$  in the lactating mammary gland could also account in large part for the substantial amount of immunoreactive and bioactive TGF $\alpha$  which we had been able to detect in human and rodent milk.

We have demonstrated that TGF $\alpha$  is consistently overexpressed to different degrees in NIH/3T3 cells that have been transformed by a number of structurally distinct retroviral oncogenes or activated cellular protooncogenes, suggesting that this growth may be an important autocrine intermediary in the cellular transformation pathway which is utilized by these genes. These observations have been extended to mouse and human mammary epithelial cells that have been transformed by two oncogenes that have been implicated in the clinical pathogenesis of human breast cancer, *c-Ha-ras* and *c-erb B-2*. Spontaneously immortalized, diploid MCF-10A human mammary epithelial cells require exogenous EGF for anchorage-dependent growth (ADG), express approximately  $3 \times 10^5$  EGF receptor sites/cell and can be transformed after transfection with a point-mutated human *c-Ha-ras* protooncogene or by overexpression of the normal *c-erb B-2* gene. Both *Ha-ras* and *c-erb B-2* transfected MCF-10A cells exhibit anchorage-independent growth (AIG) in soft agar and show a 3- to 5-fold increase in their ADG rate in serum-free medium that is devoid of exogenous EGF. In the *Ha-ras* transformed MCF-10A cells there is a reduced mitogenic responsiveness to EGF. In *Ha-ras* transformed cells, but not in the *erb B-2* MCF-10A transformants, there is a 4- to 8-fold increase in the level of TGF $\alpha$  mRNA expression and TGF $\alpha$  protein secretion, suggesting that the reduced dependency of the *Ha-ras* transformed MCF-10A mammary epithelial cells upon exogenous EGF is due in part to the enhanced production of endogenous TGF $\alpha$  and that TGF $\alpha$  is involved in the transformation pathway of mammary epithelial cells that is utilized by an activated *c-Ha-ras* gene but not by the normal *c-erb B-2* gene. However, in both *Ha-ras* and *c-erb B-2* MCF-10A transformants, mRNA expression for amphiregulin (AR), another EGF-related peptide mitogen that also functions through the EGF receptor, is enhanced by 20- to 40-fold. Likewise, in the *c-erb B-2* MCF-10A transformants but not in the *Ha-ras* transformed clones heregulin  $\alpha$  (HRG  $\alpha$ ) mRNA expression can be detected. This change is reflected by a corresponding increase in the amount of AR protein that can be detected by Western blot analysis in the *Ha-ras* and *erb B-2* MCF-10A cell lysates and by an increase in the amount of immunoreactive AR that can be detected in the cytoplasm and in the nucleus of these transformants following

immunocytochemical (ICC) localization. Exogenous AR or hepatocyte growth factor (HGF) which binds to the *c-met* receptor like EGF or TGF $\alpha$  are potent mitogens for the parental, nontransformed MCF-10A cells. This suggests that the enhanced production of AR in the *c-erb* B-2 and Ha-ras transformed MCF-10A cells may contribute to their mutated response to exogenous EGF and may also function as an autocrine growth factor for these transformants. This may be the case since specific 20-mer AR antisense phosphorothioate oligonucleotides can selectively inhibit the AIG of the Ha-ras and *erb* B-2 MCF-10A transformants. MCF-10A cells were also infected with a recombinant, replication defective amphotropic retroviral expression vector containing the human TGF $\alpha$  cDNA to assess the transforming potential of this gene. Overexpression of the TGF $\alpha$  cDNA under the transcriptional control of an internal heavy metal (cadmium)-inducible mouse MT-1 metallothionein promoter lead to a 15- to 20-fold increase in the production and secretion of TGF $\alpha$  in these cells. The TGF $\alpha$  overexpressing MCF-10A mammary epithelial cell clones formed colonies in soft agar at an efficiency equivalent to the Ha-ras or *c-erb* B-2 MCF-10A transformants, exhibited an enhanced growth rate in serum-free medium that lacks EGF and showed a diminished response to exogenous EGF. AIG in soft agar of the Ha-ras or TGF $\alpha$  transformed MCF-10A cells transformed cells could be inhibited with either an anti-EGF receptor blocking antibody or with an anti-TGF $\alpha$  neutralizing antibody demonstrating that TGF $\alpha$  is functioning through an external autocrine loop to regulate the proliferation of these transformed cells. Collectively, these results demonstrate that both TGF $\alpha$  and AR are functioning to different degrees as autocrine intermediaries in a common transformation pathway(s) that is utilized by both Ha-ras and/or *c-erb* B-2 and that the expression of three different EGF-related peptides, TGF $\alpha$ , HRG  $\alpha$  and AR, are differentially regulated by an activated Ha-ras gene and by overexpression of the *c-erb* B-2 gene in human mammary epithelial cells. Similar results have been observed in HC11 mouse mammary epithelial cells in that differentiation of these cells can be differentially modified by specific transforming genes. HC11 cells can normally be induced to differentiate in response to the lactogenic hormones, prolactin, insulin and glucocorticoids, after which they begin to synthesize the milk protein,  $\beta$ -casein. HC11 cells transformed with an activated human Ha-ras gene or by overexpression of the human TGF $\alpha$  gene are no longer able to synthesize  $\beta$ -casein in response to lactogenic hormones whereas activated rat *c-neu* (the rat homolog of *c-erb* B-2) or human *c-erb* B-2 transformed HC11 cells are still able to differentiate and synthesize  $\beta$ -casein in response to these hormones. Addition of an anti-EGF receptor blocking antibody is able to partially restore the ability of the Ha-ras, and TGF $\alpha$  transformed HC11 cells to respond to lactogenic hormones, suggesting that secreted TGF $\alpha$  is acting through an external autocrine pathway to negatively regulate  $\beta$ -casein expression through the EGF receptor. This is also supported by the observation that exogenous EGF or TGF $\alpha$  can antagonize the inductive effect of these lactogenic hormones on  $\beta$ -casein expression in the parental HC11 cells. In addition, the data suggest that activation of the EGF receptor by EGF or TGF $\alpha$  and of the *erb* B-2 receptor by the recently identified HRG, a peptide which is known to specifically activate the *c-erb* B-2 tyrosine kinase, have different effects upon mammary epithelial cell differentiation. Other growth factors may also be important in the control of mammary epithelial cell growth, differentiation, and transformation. For example, these growth factors may in fact be elaborated by mammary stromal cells and could thereby influence the behavior of adjacent mammary epithelial cells that have been sensitized to these growth factors in a paracrine manner. This may be the case since 184A1N4 (A1N4) human mammary epithelial cells or A1N4 clones that are overexpressing nuclear oncogenes such as the SV40 T or *c-myc* fail to clone in soft agar. However, we have found that A1N4 cells which overexpress either the *c-myc* or SV40 T genes can form colonies in soft agar, an index of *in vitro* transformation, specifically in response to exogenous EGF, TGF $\alpha$  or basic or acidic fibroblast growth factor (FGF) but not in response to either insulin, insulin-like growth factor-1 (IGF-I), IGF-II or platelet-derived growth factor (PDGF). An enhanced AIG response in soft agar and ADG growth of these cells in monolayer culture can also be produced by co-cultivation of the *c-myc* or SV40 T expressing A1N4 cells with primary human mammary-derived diploid fibroblasts. Conditioned medium (CM) obtained from the mammary fibroblasts can mimic these effects. CM from the mammary fibroblasts which were originally derived from reductive mammaplasty tissue contains biologically active basic FGF and the cells express specific basic and acidic FGF mRNA transcripts but not mRNA for TGF $\alpha$ . In contrast, the *c-myc* and SV40 T overexpressing A1N4 cells express TGF $\alpha$  mRNA but do not express acidic or basic FGF mRNA transcripts, demonstrating that autocrine and paracrine growth factors can equally influence the same set of target cells.

The expression of TGF $\alpha$  mRNA and TGF $\alpha$  protein in estrogen receptor (ER)-positive human breast cancer cells such as in MCF-7 or ZR-75-1 cells can be increased by growth-promoting concentrations of 17 $\beta$ -estradiol (E2), whereas in ER-negative breast cancer cell lines such as MDA-MB-231 or MDA-MB-468 cells basal levels of TGF $\alpha$  are generally higher than in the ER-positive cell lines and are insensitive to E2 regulation. Likewise, E2 can increase the level of AR mRNA in MCF-7 cells. To ascertain if E2 can directly regulate TGF $\alpha$  expression through the TGF $\alpha$  promoter/enhancer region, MCF-7 and ZR-75-1 cells were transiently transfected with plasmids containing a 1140-bp fragment of the human TGF $\alpha$  5'-flanking region ligated to either the chloramphenicol acetyltransferase (CAT) or luciferase (Luc) reporter genes. MCF-7 or ZR-75-1 cells transfected with either plasmid and subsequently treated with physiological concentrations of E2 ( $10^{-11}$  M to  $10^{-7}$  M) for 24 hrs exhibited a 10- to 100-fold increase in either CAT or Luc activity. This induction by E2 could be blocked by simultaneous treatment of the cells with a 10-fold higher concentration of the antiestrogens, tamoxifen or droloxifene. E2 was unable to affect CAT or Luc activity following transfection of these reporter plasmids into MDA-MB-231 cells. These results demonstrate that this inductive effect through the TGF $\alpha$  5'-flanking region is an ER mediated response. Using various sized fragments of the TGF $\alpha$  5'-flanking region (2,800 to 77 bp), we were able to establish the presence of at least two 13-bp imperfect palindromic sequences that could function as potential estrogen response elements (EREs) and that are within -370 bp from the transcription initiation start site. To ascertain if E2-induced proliferation could be attenuated by blocking the expression of endogenous TGF $\alpha$ , MCF-7, ZR-75-1, MDA-MB-468 or ER-negative HS-578T cells were infected with a replication defective, recombinant amphotropic retrovirus containing a 406-bp fragment of the coding region of the human TGF $\alpha$  gene oriented in the reverse 3' to 5' direction and under the transcriptional control of an internal MT-1 promoter in order to generate a specific inducible TGF $\alpha$  antisense mRNA. Infected MCF-7 or ZR-75-1 cells expressed the TGF $\alpha$  antisense mRNA, exhibited a 60% to 70% reduction in E2-stimulated TGF $\alpha$  mRNA expression and TGF $\alpha$  protein production and a 45% to 70% reduction in E2-stimulated ADG or AIG after induction of the antisense mRNA vector with CdCl $_2$ . A similar inhibitory response on the basal growth rate was observed in MDA-MB-468 cells that were expressing the TGF $\alpha$  antisense mRNA but not in antisense infected ER-negative HS-578T breast cancer cells which do not express endogenous TGF $\alpha$ . In addition, in infected MCF-7 cells expression of the TGF $\alpha$  antisense mRNA had no effect on E2-induced progesterone receptor (PgR) expression or on IGF-I stimulated proliferation.

In primary human breast tumors, an association exists between high EGF receptor expression and an absence of ERs. To determine if there is any functional relationship between these two phenotypes, ER-positive ZR-75-1 breast cancer cells that express low levels of EGF receptors, approximately  $2 \times 10^4$  EGF receptor sites/cell, were transfected with an Ha-MSV expression vector plasmid containing the human EGF receptor gene and a selectable neomycin (*neo*) marker. Several *neo*-resistant ZR-75-1 clones were selected and found to express up to  $1.2 \times 10^6$  EGF receptor sites/cell. These EGF receptor overexpressing clones possessed functionally normal EGF receptors since they could be autophosphorylated in response to exogenous EGF and could transphosphorylate the p185<sup>erbB-2</sup> protein after EGF treatment. No change in either the number or affinity of ERs were observed in these EGF receptor overexpressing clones as compared to the *neo* transfected or parental ZR-75-1 cells. More importantly, E2 was still capable of stimulating the ADG and AIG of these clones and could induce PgRs to the same degree as that observed in the *neo* transfected or parental ZR-75-1 cells, thereby demonstrating that an increase in EGF receptor expression *per se* may be necessary but not entirely sufficient to induce an estrogen-independent phenotype in human breast cancer cells.

TGF $\alpha$  is one of several EGF-related proteins that may be involved in regulating the proliferation of tumor cells through an autocrine and/or juxtacrine mechanism. AR and cripto-1 (CR-1) are two other members of the EGF gene family. Whereas AR can function as a mitogen for mammary epithelial cells and can bind to the EGF receptor, it is not yet known whether CR-1 is a growth factor and has a specific membrane-associated receptor, since a chemically synthesized or recombinant protein has not yet been produced. Nevertheless, overexpression of the human CR-1 gene using an RSV retroviral expression vector in mouse NIH-3T3 fibroblasts and in mouse NOG-8 or human MCF-10A mammary epithelial cells can lead to the *in vitro* transformation of these cells as assessed by their ability to form foci in monolayer culture or to form colonies in soft agar at

a frequency equivalent to Ha-*ras* transformed cells. In addition, specific mRNA transcripts for TGF $\alpha$  (4.8kb), AR (1.4kb) and CR-1 (2.2kb) are expressed in a majority of human colon cancer cell lines. CR-1 and AR mRNAs are also expressed in 50% to 70% of 78 primary or metastatic colorectal tumors, whereas only 2% to 7% of 38 noninvolved, adjacent dysplastic colon tissues or normal liver expressed these transcripts. ICC localization studies demonstrated that AR and CR-1 proteins could be detected in the colorectal tumor cells and not in the surrounding stroma. More importantly, 50 to 80% of human colorectal tumors that were examined expressed either immunoreactive AR or CR-1, respectively. AR expression was generally associated with more well-differentiated tumors whereas CR-1 expression was independent of the degree of tumor cell differentiation. AR but not CR-1 is also expressed in normal colonic mucosa obtained from noncancer patients. Approximately 60% of polyps expressed AR and CR-1. However, CR-1 was generally expressed at a lower frequency in more benign tubular adenomas as compared to more aggressive tubulovillous adenomas. Approximately 50% of reduction mammoplasty breast tissue samples and primary human breast tumors were found to express AR mRNA. Immunoreactive AR and CR-1 could be detected in nearly 70% of 26 infiltrating ductal breast carcinomas at a level which exceeded the level of staining observed in adjacent noninvolved mammary epithelium, suggesting that these two EGF-related peptides may be important as potential tumor markers and may also perform some biological role in the pathogenesis of clinical colon and breast cancer, possibly as autocrine growth factors.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CB 04829-18 LTIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## 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)

PI:	Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBDC, NCI
Others:	Craig Cropp	Senior Staff Fellow	LTIB, DCBDC, NCI
	Zong-mei Sheng	Visiting Fellow	LTIB, DCBDC, NCI
	Francesca Diella	Visiting Fellow	LTIB, DCBDC, NCI
	Shukichi Miyazaki	Visiting Fellow	LTIB, DCBDC, NCI

## COOPERATING UNITS (if any)

Dr. R. Lidereau, Centre Rene Huguenin, St. Cloud, France; Dr. D. Liscia, S.Giovanni Hospital, Torino, Italy; Dr. Patricia Steeg, NCI; Dr. R. White, Howard Hughes Med. Ctr. U. of Utah; Dr. B. Ponder, Cambridge Univ., Cambridge, UK; Drs. Marchetti and Bistocchi, Univ. of Pisa, Pisa Italy; Drs. Marchetti and Bistocchi, (Cont. page 2)

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Oncogenetics Section

## INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 3.5

PROFESSIONAL: 3.5

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.)

We have undertaken an ongoing program that is aimed at determining, on a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant association with the patients history, characteristics of the tumor, and the patients prognosis. The most frequent type of mutation is loss of heterozygosity (LOH) at specific regions of the cellular genome in tumor DNA. In previous studies we have found LOH on chromosomes 1p, 1q, 3p, 7q, 11p, 13q, 17p, 17q, and 18q. We have begun to focus on potential target genes affected by LOH. Our current results show that the p53 gene on chromosome 17p13 is altered in 29% of the primary breast tumor DNAs (n=121) examined by the PCR-SSCP technique. The location of the mutations within the gene was evenly distributed in exons 5 through exon 8. We have found that there is a significant association (p=0.003) between tumors having a p53 mutation and those having a high proliferative index as measured by BUdR incorporation. Furthermore, this association appears to primarily reflect those tumors having a mutation in either exon 5 (p=0.0002) or exon 6 (p=0.05). There is a body of evidence suggesting that p53 mutations are involved in the process of immortalization of mammalian cells. In other studies we have found that the MCF10A and A1N4 "normal" human breast cell lines, although immortalize for growth in tissue culture, have an unaltered p53 gene. This suggests that either there are other mechanisms by which p53 is inactivated or there are other mutations, independent of those in p53, which cause immortalization of cells in culture. We have previously reported that the NME1 gene on chromosome 17q21 is frequently affected by LOH in primary breast tumors. Others have shown that loss of NME1 expression in breast tumors is associated with a poor prognosis for the patient. We have confirmed this finding. In addition, we have found trend for an association between loss of expression and LOH of the NME1 gene. This association was not perfect, however, suggesting that there could be a closely linked target gene for LOH in this region of chromosome 17.

Cooperating Units (Cont.) University of Pisa, Pisa Italy; Dr. H. Nevanlinna, Helsinki University Central Hospital, Helsinki, Finland

#### Major Findings:

Several years ago we initiated a major effort to survey at a molecular level the genome in primary human breast tumors with the aim to identify frequently occurring somatic mutations of specific genes or alterations of specific regions of the cellular genome. The goal of these studies was to determine whether specific mutations have a significant association with biochemical or morphologic characteristics of the tumor, aspects of the patients history, or the subsequent course of the disease. Using anonymous recombinant DNA probes which detect sequences in the genome that are highly polymorphic and whose chromosomal location has been determined we systematically examined each chromosomal arm to define the "allele types" of the tumor DNAs. We have found, to date, twelve mutations that frequently occur at different chromosomal sites in our panel of primary human breast tumors. Three of these represent amplification of cellular proto-oncogenes (*MYC*, *ERBB2*, and *INT2*) while the remainder are regions of the cellular genome affected by LOH (chromosomes 1p, 1q, 3p, 7q, 11p, 13q, 17p, 17q, and 18q). We have begun to shift the focus of our analysis from allele typing tumor DNAs to defining the target genes in the regions of the genome affected by LOH.

#### The *TP53* gene

The *TP53* gene is located on chromosome 17p13 and encodes a protein that is involved in regulating the transition from the G1 to S phase of the cell cycle. In a earlier pilot study of 30 primary breast tumors we demonstrated that the polymerase chain reaction-single strand conformation polymorphism analysis (PCR-SSCP) technique was the most sensitive approach for detecting mutations on the *TP53* gene. This study has now been expanded to include a total 121 tumors which had been typed for their proliferative index. The overall frequency of *TP53* mutations was 29% and their location within the gene was evenly distributed between exons 5 through exon 8 (exon 5, 10%; exon 6, 9.9%; exon 7, 7.1%, exon 8, 5.5%). In general, there is a significant association ( $p=0.003$ ) between tumors having a high proliferative index and those having a *TP53* mutation. However, stratification of the data based on the exon in which the mutation occurred showed that the association with proliferative index appears to reflect primarily tumors having a mutation in either exon 5 ( $p=0.0002$ ) or exon 6 ( $p=0.05$ ), but not exons 7 or 8. Since both LOH at *pYnz22.1/pYnh33.7* (REF) and *TP53* mutations are significantly associated with tumors having a high proliferative index, we questioned whether these mutations were associated with one another. Mutations of the *TP53* gene had a significant association ( $p=0.0038$ ) with LOH of the remaining *TP53* allele, whereas no association was observed between these *TP53* mutations and LOH at other loci on chromosome 17p. These data taken together suggest that there are two loci on chromosome 17p, each of which when mutated are independently associated with tumors having a high proliferative index. It has been reported that tumors having a high proliferative index are also the more aggressive tumors and this characteristic is predictive of a poor prognosis for the patient. In the case of our tumor panel, not enough time has elapsed since surgery to determine whether *TP53* exon 5 and exon 6 mutations are predictive of the patients prognosis. However our results are consistent with two other studies, in which *TP53* protein accumulation was ascertained in paraffin-embedded sections of breast tumor biopsies by immunohistochemical techniques. In these studies a significant association was found between *TP53* positive tumors and decreased disease-free interval and overall survival.

There is a body of evidence supporting the thesis that mutations within the *TP53* gene are frequently involved in the process of immortalization of mammalian cells. Genomic and cDNA clones of mutant forms of the human *TP53* gene have been shown to immortalize primary rodent embryo fibroblasts in culture and to cooperate with an activated ras gene in the transformation of

primary rat embryo fibroblasts. The WT *TP53* gene does not possess these biological properties. The effect of *TP53* expression on cell immortalization has been studied mainly in primary cultures of rodent embryo fibroblasts. In contrast, little is known about its function in mammary epithelial cells. There is some indirect evidence suggesting that loss of WT *TP53* function may be important for epithelial cells to escape senescence. For example, human papilloma virus DNA (HPV16 or 18) or SV40 large T oncogene can immortalize human epithelial cells in culture. Both of these viruses encode oncoproteins which bind to and inactivate the *TP53* and *RB1* tumor suppressor proteins. However, the precise action of *TP53* alone has not been determined.

We have examined the *TP53* gene in the nontransformed cell lines MCF-10A and A1N4 and the human breast cancer cell lines MDA-MB-231 and ZR-75-1. DNAs were screened by the PCR/SSCP methods for point mutations within the "hot spot" region corresponding to exons 4–9 of the *TP53* gene. Radiolabeled PCR fragments were electrophoresed in a nondenaturing polyacrylamide gel, and mutant sequences were recognized by a shift in mobility of an allelic fragment. The *TP53* gene was found to be mutated in MDA-MB-231 cell line, at exon 8, (Fig. 2) as previously reported by others. The cell line ZR-75-1 also showed a mutation in exon 4 (Fig. 2). We observed no mutations in exons 4–9 in MCF-10A and A1N4 DNA. In neither MCF10A nor in A1N4 cells could *TP53* protein be detected by immunohistochemical or Western blot analysis. It should be noted that both MCF10A and A1N4 cells unlike the breast cancer cell lines are completely dependent upon EGF and IGF-1 for growth and maintenance in culture. In the absence of a *TP53* missense mutation, the WT *TP53* protein could still be inactivated by a variety of other mechanisms. For instance, the expression of a second gene whose protein product binds to and inactivates the WT *TP53* protein may be activated in these cells, resulting in an epigenetic inactivation of the *TP53* function. Alternatively, mutations could exist outside the region that we have analyzed, although more than 98% of the mutations described so far occur within exons 4–9. Another possibility is that *TP53* inactivation may not be required for immortalization of human mammary epithelial cells. In this regard, MCF-10A cells that have been transfected and/or infected with different activated oncogenes are able to form colonies in agar and are less dependent upon exogenous growth factors than the parental MCF-10A cells, but are still not tumorigenic in nude mice. Inactivation of the MCF-10A WT *TP53* gene may be a necessary prerequisite for the acquisition of a fully transformed phenotype. Conversely, if an epigenetic inactivation of the WT *TP53* protein occurs in these nontransformed mammary epithelial cells, we might expect a suppression of their growth after introduction and over expression of the WT *TP53* gene

### The *NME1* gene

We have reported that chromosome 17q21 is also frequently affected by LOH in primary breast tumors [65]. The *NME1* gene, located at 17q21, was affected by LOH in 64% of the informative tumors [79]. Further allele typing of closely linked markers in tumors having LOH at *NME1* showed that the gene lies within a defined region of 17q21 that is frequently affected by LOH. The *NME1* gene encodes a nucleoside diphosphate kinase and is a candidate suppressor protein for metastasis. Previous studies by others have shown that the absence of the *NME1* RNA or protein in primary breast tumors is associated with a poor prognosis for the patient. To determine the relationship between LOH at *NME1* and loss of *NME1* protein expression, we analyzed a panel of primary breast tumors for mutations of the *NME1* gene, *NME1* protein levels by immunohistochemistry, and the patients clinical course. We were able to confirm that patients whose tumors had either focal or diffuse low staining with an monoclonal antibody prepared against the *NME1* protein had a significantly ( $p=0.01$ ) poorer prognosis, as defined by metastasis-free survival curves, than patients with tumors having a uniform high level of staining. In addition we observed a significant trend ( $p=0.044$ ) for an association between tumors which have lost one allele of the *NME1* gene by LOH and those tumors having either focal or generalized loss of the *NME1* protein in the tumor cells. This association, however, was not perfect. For instance in several tumors having LOH at the *NME1* gene, there was no evidence of loss of *NME1* protein accumulation. Moreover, LOH at *NME1* was not associated with the patients prognosis. This

suggests that a closely linked gene may be the target for LOH in some tumors in which the expression of *NME1* is unaffected. We are currently investigating the possibility that in those breast tumors where there is a loss of expression of *NME1* that in addition to LOH of one allele, the other allele has a point mutation which blocks expression.

One of the limiting factors in designing studies to determine whether specific mutations are prognostically significant is the relative scarcity of frozen primary breast tissue and matching normal tissue from surgeries performed five to ten years ago. An obvious alternative source could be formalin-fixed, paraffin-embedded (FFPE) tissues. Previous reports have described methods for extraction of DNA from neutral FFPE tissues. Although the recovered DNA was not intact, it was suitable for several applications in molecular biology, including Southern blot analysis. Unfortunately, in most pathology departments tissues are routinely fixed with acid formalin, a procedure which results in tissue blocks with a marked degradation of nucleic acids. Nevertheless, our data show that DNA extracted from acid FFPE tissues can be used for a mobility shift analysis in a non denaturing polyacrylamide gel, after PCR amplification. Our method is easy and reproducible, allowing several clinico-pathological applications e.g. a) systematic retrospective studies are possible, since non buffered FFPE human tissues are available in most pathology departments and stored routinely for many years; b) unusual tumors as well as early stage premalignant lesions, infrequently available as fresh specimens, can be analyzed. In addition, paraffin sections can be stained and microdissected before PCR amplification in order to separate tumor cells from stromal and inflammatory cell populations. This can be useful when the method is applied to those cancer types (e.g. breast, lung) that often contain populations of infiltrating tumor cells surrounded by a large amount of nonneoplastic tissue.

#### Publications:

Merlo GR, Bernardi A, Diella F, Venesio T, Cappa APM, Callahan R, and Liscia DS. In primary human breast cancer mutations in exons 5 and 6 of the p53 gene identify tumors with high S-phase index. *Int. J. Cancer* In Press.

Marchetti A, Merlo G, Buttitta F, Callahan R, Bistocchi M, and Squartini F. Detection of DNA mutations in formalin-fixed paraffin-embedded archival tumor specimens by polymerase chain reaction-single strand conformation polymorphism analysis. *CancerDetect. & Prevent.* In Press

Callahan R, Cropp C, Merlo GR, Diella F, Venesio T, Lidereau R, Cappa APM and Liscia DS. Cellular and molecular heterogeneity of breast cancer cells. *Clinica Chimica Acta* In Press.

Diella F, Normanno N, Merlo GR, Salomon DS, and Callahan R. Absence of p53 point mutations in nontransformed human mammary epithelial cell lines. *Life Sci. Advances* In Press.

Marchetti A, Buttitta F, Merlo G, Diella F, Pellegini S, Pepe S, Macchiarini P, Angeletti CA, Callahan R, Bistocchi M, and Squartini F. p53 alterations in non small cell lung cancers correlate with metastatic involvement of hilar/mediastinal lymph-nodes and advanced stages of disease. *Cancer Res.* In Press.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CB 05148-14 LTIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## 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)

PI: Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBDC, NCI
Others: Gilbert Smith	Research Biologist	LTIB, DCBDC, NCI
Daniel Gallahan	Senior Staff Fellow	LTIB, DCBDC, NCI
Francesca Diella	Visiting Fellow	LTIB, DCBDC, NCI
Edith Kordon	Visiting Fellow	LTIB, DCBDC, NCI
Shukichi Miyazaki	Visiting Fellow	LTIB, DCBDC, NCI

## COOPERATING UNITS (if any)

Drs. Antonio Marchetti, Univ. of Pisa, Pisa, Italy; Drs. Gleen Merlino and Chameli Jhappen, NCI; Dr. Lothar Hennighausen, NIDDK; Dr. Richard Hodes, NCI.

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Oncogenetics Section

## INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 5.5

PROFESSIONAL: 4.5

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 mammary tumor system has been useful both in the identification and characterization of genes involved in the development and transformation of mammary epithelium. Further characterization of the Notch related Int-3 gene reveals that it encodes a 6.2kb message expressed at a low level in almost all adult tissues and appears to be transcriptionally active as early as day 8 in developing embryos. We have demonstrated in the "normal" HC11 mouse mammary cell line that the activated Int-3 gene transforms these cells to anchorage independence while maintaining the normal growth kinetics suggesting a mode of transformation though direct cell-cell interactions. This is in contrast to what we had previously observed with /int-2 transformed HC-11 cells. In addition, expression of activated Int-3 abrogates the ability of HC11 cells to express beta-caesin in response to lactogenic hormones. The HC11 cells, while maintaining certain characteristics of normal cells, are immortal. Further analysis of this cell line reveals that one allele of the Trp53 gene contains a microdeletion of seven codons in exon 5 and the other allele contains a missense mutation in exon 6. Analysis of one of the MMTV induced mammary hyperplastic outgrowth (HOG) lines in CZECHII mice has led to the identification of a new common insertion site for MMTV. We have named this new site /nt-6. It was found to be rearranged by MMTV, four independent tumors. Preliminary data indicated that the gene encodes a 1.4 kb RNA transcript and is located on chromosome 15 in the mouse. Each of the endogenous superantigens analyzed to date is determined by one or more MMTV proviral genomes. To assess the ability of self ligands other than MMTV to mediate the deletion of specific populations of T-cells during T-cell development, the T-cell receptor beta chain (Tcrb-V) repertoire was characterized in CZECHII mice. These mice lack endogenous MMTV proviral genomes. Our results showed that although CZECHII mice express some Tcrb-V products at frequencies lower than those observed in nondeleting conventional mouse strains, these instances do not however appear to involve negative selection. These results are consistent with the conclusion that endogenous superantigens are uniquely the products of integrate proviral genomes.

**Major Findings:****Characterization of *Int-3***

HC11 cells are incapable of anchorage independent growth in the presence or absence of dexamethasone. In earlier studies we have shown that a restriction fragment containing a portion of the MMTV ENV gene, all of the MMTV LTR and the transcriptionally activated portion of the *Int3* gene can, in the presence of dexamethasone, induce HC11 cell growth in soft agar. Paradoxically, we have now found that similar treatment of HC11/MMTV LTR-*Int-3* cells in monolayer culture does not alter the doubling time of the cells. This suggests that the effect of activated *Int-3* expression may be at the level of cell-cell interactions rather than cellular proliferation. In addition, the capability of HC11 cells to produce b-caesin in response to lactogenic hormones is abrogated in the dexamethasone treated HC11/MMTV LTR-*Int3* cells. Thus expression of the activated *Int3* gene can both perturb the growth properties of mammary epithelial cells as well as affect their ability to differentiate.

Mutations in the *Trp53* gene are frequently found in primary human breast tumors. To understand the role of *Trp53* in the context of the multistep accumulation of mutations in breast cancer, a model of nontransformed mammary cells was sought. We have recently detected, by immunoprecipitation with PAb421, the accumulation of the *Trp53* protein in HC11 cells. This finding suggested that the *Trp53* protein had an extended half-life that was likely due to mutations. Nucleotide sequence analysis of *p53* cDNA from HC11 cells revealed two mutations: a missense mutation at codon 138, substituting a Trp residue for a Cys residue, and a microdeletion of seven amino acids from codon 123 to 130 of exon 5. The latter results from an intronic mutation of the splice acceptor site at the intron 4/exon 5 junction. The two mutations affect different alleles since no wild type allele was found. HC11 cells, therefore, provide an ideal *in vitro* model for assessing the cooperative action of other mutations in mammary tumorigenesis.

We have developed several MMTV induced mammary hyperplastic outgrowth (HOG) lines in the CZECHII mouse strain. DNA from one of these, designated CZZ-1, was found by Southern blot analysis to contain three integrated MMTV proviral genomes. These proviral genomes were also present in primary tumors which arose from within the HOG as well as metastatic lesions in the lung. Moreover, many of these tumors had additional integrated MMTV genomes, raising the possibility that they contributed to tumor progression by activating additional genes. Since we found that none of the known common insertion sites for MMTV were rearranged in the CZZ-1 HOG, recombinant clones were obtained of each of the host-MMTV junction restriction fragments. Subclones of the host sequences were used as probes to screen Southern blots of independent mammary tumor DNAs for evidence of MMTV-induced rearrangements. Using this approach, the host sequences flanking one of the MMTV proviruses in the CZZ-1 HOG detected MMTV-induced rearrangements in three additional independent tumors. Thus these host sequences define a new common insertion site for MMTV which we have called *Int-6*. The *Int-6* locus was mapped in collaboration with Dr. C. Kozac (LMM, NIAID) to chromosome 15 using (NFS X *M. musculus*) X *M. musculus*; (NFS X *M. spretus*) X *M. spretus*; and (NFS X *M. spretus*) X C58 genetic backcrosses and is located 10 cM centromeric of *Myc*. A 1.4 kb RNA species was detected using a recombinant *Int-6* genomic DNA probe in cellular RNA from a tumor in which the locus was rearranged by MMTV.

A class of superantigens has recently been described for which T-cell recognition is dependent predominantly on the V region of the T-cell receptor  $\beta$  (Tcr) chain. When expressed as endogenously encoded self antigens in the mouse, superantigens mediate Tcrb-V-specific deletion during T-cell development. Each of the endogenous superantigens analyzed to date is determined by one or more MMTV proviral genomes. To assess the ability of self ligands other than MMTV to mediate Tcrb-V-specific negative selection, the Tcrb-V repertoire was characterized in CZECHII mice which lack endogenous MMTV proviral genomes. Our results showed that although CZECHII mice express some Tcrb-V products at frequencies lower than those observed in nondeleting conventional mouse strains, these instances do not appear to

involve negative selection. These findings are therefore consistent with the conclusion that endogenous superantigens are uniquely the products of integrated MMTV sequences.

Publications:

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CB 05216-22 LTIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Site-Selective cAMP Analogs and Antisense Oligonucleotides as Antineoplastics and Chemopreventives

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

PI: Yoon S. Cho-Chung	Chief	LTIB, DCBDC, NCI
Others: Timothy Clair	Chemist	LTIB, DCBDC, NCI
Alfredo Budilon	Visiting Fellow	LTIB, DCBDC, NCI
Anna Cereseto	Visiting Fellow	LTIB, DCBDC, NCI
María Nesterova	Visiting Fellow	LTIB, DCBDC, NCI

## COOPERATING UNITS (if any)

Dr. S. Bates, Medicine Branch, NCI, Bethesda, MD; Dr. W.R. Miller, U. of Edinburgh, Scotland; Dr. A. Pinto, Centro di Riferimento Oncologico, INRCCS, Aviano, Italy; Dr. G. Tortora, U. of Naples Medical School II, Naples, Italy; Dr. G. Weber, Indiana School of Medicine, Indianapolis, IN

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Cellular Biochemistry Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 2.0

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 unrounded type. Do not exceed the space provided.)

The use of site-selective cAMP analogs greatly advanced our understanding of the mechanism of cAMP action in growth control. It was discovered that site-selective cAMP analogs can act as novel biological agents capable of inducing growth inhibition and differentiation in a broad spectrum of human cancer cell lines, including carcinomas, sarcomas, and leukemias, without causing cytotoxicity. 8-CI-cAMP, the most potent site-selective cAMP analog, was selected as a preclinical Phase I antineoplastic agent of the National Cancer Institute (January 27, 1988). It was the first introduction of a cAMP analog into clinical testing in over 30 years of cAMP research. Significantly, this was the first demonstration that a cAMP analog can induce its biological effect at micromolar concentrations-the physiological concentration of cAMP, as opposed to the millimolar pharmacological or cytotoxic concentrations of cAMP analogs reported in all previous literature. The discovery rendered a critical assessment that the potency of a cAMP analog in growth inhibition depends on the analog's ability to selectively modulate the RI and RII regulatory subunits of cAMP-dependent protein kinase, precisely, down-regulation of RI with up-regulation of RII leading to the restoration of the normal balance of these cAMP transducing proteins in cancer cells. The use of antisense strategy and retroviral vector-mediated gene transfer technology provided direct evidence that two isoforms, the RI and RII regulatory subunits of cAMP-dependent protein kinase, have opposite roles in cell growth and differentiation; RI being growth stimulatory while RII is a growth-inhibitory and differentiation-inducing protein. As RI expression is enhanced during chemical or viral carcinogenesis, in human cancer cell lines, in primary human tumors, and in multidrug-resistant (MDR) cancer cells as opposed to non-MDR parental cells, it is a target for cancer diagnosis and therapy. 8-CI-cAMP and RI antisense oligodeoxynucleotide, those that effectively down-regulate RI and up-regulate RII, provide new approaches toward differentiation therapy and chemoprevention of cancer. 8-CI-cAMP is now in Phase I clinical studies at several Institutes where Y.S. Cho-Chung is collaborating as the consultant.

## Major Findings

Inhibition of the self-renewal capacity of blast progenitors from acute myeloblastic leukemia patients by site-selective 8-CI-cAMP. The physiologic balance between the two regulatory subunit isoforms, RI and RI $\beta$ , of cAMP-dependent protein kinase is disrupted in cancer cells; growth arrest and differentiation of malignant cells can be achieved when the normal ratio of these intracellular signal transducers of cAMP is restored by the use of site-selective cAMP analogs. In this study we evaluated the effects of the site-selective cAMP analog 8-CI-cAMP on clonogenic growth of blast progenitors from 15 patients with acute myeloblastic leukemia and 3 patients affected by advanced myelodysplastic syndrome. Leukemic blast progenitors undergo terminal divisions, giving rise to colonies in methylcellulose. The self-renewal capacity of blast progenitors is conversely reflected in a secondary methylcellulose assay after exponential growth of clonogenic cells in suspension cultures. In all the samples tested, 8-CI-cAMP, at micromolar concentrations (0.1-50  $\mu$ M), suppressed in a dose dependent manner both primary colony formation in methylcellulose and the recovery of clonogenic cells from suspension culture. Strikingly, in the samples from the entire group of patients, 8-CI-cAMP was more effective in inhibiting the self-renewing clonogenic cells than the terminally dividing blast cells ( $P = 0.005$ ). In addition, in four out of six cases studied, 8-CI-cAMP was able to induce a morphologic and/or immunophenotypic maturation of leukemic blasts. An evident reduction of RI levels in fresh leukemic cells after exposure to 8-CI-cAMP was also detected. Our results showing that 8-CI-cAMP is a powerful inhibitor of clonogenic growth of leukemic blast progenitors by primarily suppressing their self-renewal capacity indicate that this site-selective cAMP analog represents a promising biological agent for acute myeloblastic leukemia therapy in humans.

An antisense oligodeoxynucleotide that depletes RI $\alpha$  subunit of cyclic AMP-dependent protein kinase induces growth inhibition in human cancer cells. Enhanced expression of the RI $\alpha$  subunit of cyclic AMP-dependent protein kinase type I has been correlated with cancer cell growth. We provide evidence that RI $\alpha$  is a growth-inducing protein that may be essential for neoplastic cell growth. Human colon, breast, and gastric carcinoma and neuroblastoma cell lines exposed to a 21-mer human RI $\alpha$  antisense phosphorothioate oligodeoxynucleotide (S-oligodeoxynucleotide) exhibited growth inhibition with no sign of cytotoxicity. Mismatched sequence (random) S-oligodeoxynucleotides of the same length exhibited no effect. The growth inhibitory effect of RI $\alpha$  antisense oligomer correlated with a decrease in the RI $\alpha$  mRNA and protein levels and with an increase in RI $\beta$  (the regulatory subunit of protein kinase type II) expression. The growth inhibition was abolished, however, when cells were exposed simultaneously to both RI $\alpha$  and RI $\beta$  antisense S-oligodeoxynucleotides. The RI $\beta$  antisense S-oligodeoxynucleotide alone, exhibiting suppression of RI $\beta$  along with enhancement of RI $\beta$  expression, led to slight stimulation of cell growth. These results demonstrate that two isoforms of cyclic AMP receptor proteins, RI $\alpha$  and RI $\beta$  are reciprocally related in the growth control of cancer cells and that the RI $\alpha$  antisense oligodeoxynucleotide, which efficiently depletes the growth stimulatory RI $\alpha$ , is a powerful biological tool toward suppression of malignancy.

Role of type I regulatory subunit (RI) of cAMP-dependent protein kinase (PKA) in multidrug resistance (MDR) of cancer cells. Enhanced expression of RI $\alpha$  of PKA is consistently observed in MDR cancer cell lines as compared to non-MDR parent cell lines. 8-CI-cAMP exerts growth inhibition, suppression of MDR-1 expression, and increase of drug sensitivity of MDR cells. In this study we examined the role of RI $\alpha$  and catalytic (C) subunits of PKA in the growth and MDR-1 expression of MDR cell lines, HL-60, KB-V1, 3T3-MDR, and MCF-7TH. We measured RI $\beta$  by immunoblotting and C by PKA activity ratio, which measures the degree of free C subunit release. 8-CI-cAMP treatment sharply reduced RI $\alpha$  levels to 40 and 5% of the control values by 48 and 72 hours. The PKA activity ratio, however, did not change up to 48 hours when both holoenzyme and free C activity decreased to 50% of the control values. Exposure of cells to 21-mer RI $\alpha$  antisense S-oligonucleotide (6  $\mu$ M) for 4-5 days brought about marked growth inhibition. RI $\beta$  antisense or random S-oligomers had no effect. The effect of RI $\alpha$  antisense oligonucleotide on MDR-1 expression is being assessed. These

results suggest that R1 $\alpha$  but not C subunit of PKA may be causally related to the multidrug resistance of cancer cells.

Different expression of mRNA for R1 $\alpha$  subunit of cAMP-dependent protein kinase (PKA) between breast and colorectal carcinomas and normal counterparts. Enhanced expression of type I regulatory subunits (R1 $\alpha$ ) of cAMP-dependent protein kinase (PKA) has been found in primary colon and breast carcinomas as compared to the normal counterparts. In this study, using Northern blot analysis we have examined specimens of breast and colorectal carcinomas and normal breast and colorectal tissues. In breast carcinomas, 4.4, 2.6, 1.9, and 1.0 kb R1 $\alpha$  mRNA were detected, whereas in normal breast, only 4.4 kb R1 $\alpha$  mRNA was detected. The expression of four species of R1 $\alpha$  mRNA in breast carcinomas varied quantitatively and qualitatively among the tumors examined: some tumors expressed 2.6, 1.9, and 1.0 kb bands with no 4.4 kb band, while others expressed only 4.4 kb mRNA as did normal breast. These variations in the expression of R1 $\alpha$  mRNA species were not related to the degradation of mRNA. Interestingly, tumors expressing only 4.4 kb band had a low [3H]-cAMP- binding activity as normal breast. In the majority of colorectal carcinomas examined, enhanced levels of 4.4 kb R1 $\alpha$  mRNA were detected as compared to the adjacent normal colorectal tissues. Studies are underway to determine whether R1 $\alpha$  mRNA expression can be of value for the diagnosis and prognosis of breast and colon cancers.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 CB 08281-11 LT1B

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Mechanism of cAMP Action in Growth Control, Differentiation, and Gene Regulation

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

PI: Yoon S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBDC, NCI
Others: Alfredo Budillon	Visiting Fellow	LTIB, DCBDC, NCI
Timothy Clair	Chemist	LTIB, DCBDC, NCI
Christian Rohlf	Special Volunteer	LTIB, DCBDC, NCI
Anna Cereseto	Visiting Fellow	LTIB, DCBDC, NCI
Maria Nesterova	Visiting Fellow	OIA, NCI

## COOPERATING UNITS (if any)

Dr. R.I. Glazer, Dept. of Pharmacology, Georgetown Univ., Washington, D.C.; Drs. K. Cowan and S. Bates, Medicine Branch, NCI, Bethesda, MD; Dr. Doskeland, Univ. Bergen, Norway; and Drs. M. Ellis and K. Cullen, Lombardi Cancer Center, Georgetown Univ., Washington, D.C.

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Cellular Biochemistry Section

## INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 2.5

PROFESSIONAL: 1.5

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 striking growth inhibitory effect of 8-CI-cAMP has been related to its selective binding and activation of protein kinase isozymes: It binds to RI with a high affinity for Site B but with a low affinity for Site A, keeping type II protein kinase in the holoenzyme form, while binding with moderately high affinity for both Site A and Site B to RI, facilitating dissociation of the RI subunit and down-regulation of type I protein kinase. The growth inhibition induced by 8-CI-cAMP brought about various effects among the cell lines tested, including the suppression of oncogenes and transforming growth factor  $\alpha$  (TGF $\alpha$ ), and morphological changes, differentiation, and reverse transformation. Despite the appearance of markers of mature phenotype and definitive growth arrest, the 8-CI-cAMP-treated leukemic cells exhibited no change in the cell cycle phase. 8-CI-cAMP therefore produces growth inhibition while allowing the cells to progress through their normal cell cycle, albeit at a slower rate, and this may lead to eventual restoration of a balance between cell proliferation and differentiation in cancer cells. Thus, unlike cytotoxic drugs, 8-CI-cAMP does not act to prevent mitosis but acts to alter the growth ratio, the ratio of cell births to cell deaths, via restoration of the RI/RII balance in cancer cells. The cellular events underlying growth inhibition and differentiation of cancer cells induced by 8-CI-cAMP include a rapid nuclear translocation of RII $\beta$ , and such translocation of RII $\beta$  into the nucleus correlates with an increase in transcription factors in cancer cells that bind specifically to cAMP response element (CRE). Thus, the mechanism of action of 8-CI-cAMP in the suppression of malignancy may involve the restoration of normal gene transcription in cancer cells where the RII $\beta$  cAMP receptor plays an important role. By the use of site-directed mutagenesis technique, the structure-function analysis of RI and RII is currently underway. The RI and RII are distinguished by their autophosphorylation and nuclear translocation properties. RII has an autophosphorylation site at a proteolytically sensitive hinge region around the R and C interaction site while RI has a pseudo-phosphorylation site. The RII but not the RI contains a nuclear location signal, K K R K. The autophosphorylation and nuclear location sequences are either point-mutated in RII $\beta$  of introduced into RI $\alpha$  to specifically assess the role of these sequences in the growth regulatory function. These studies contribute to understanding the mechanism of cAMP control cell growth and differentiation and provide new approaches to the treatment of cancer.

### Major Findings

8-CI-cAMP induces truncation and down-regulation of the R1 $\alpha$  subunit and up-regulation of the R11 $\beta$  subunit of cAMP-dependent protein kinase leading to type II holoenzyme-dependent growth inhibition and differentiation of HL-60 leukemia cells. 8-CI-cAMP, a site-selective cAMP analog, induces growth inhibition in a variety of cell types of human cancer cell lines. This inhibitory effect of 8-CI-cAMP was related to its ability to differentially regulate type I versus type II cAMP-dependent protein kinase. In the present study we demonstrated a unique mechanism of action of 8-CI-cAMP in the regulation of these kinase isozymes in HL-60 human promyelocytic leukemia cells. High-performance liquid chromatography (HPLC) resolved various isoforms of protein kinase present in HL-60 cells. In control cells, type I protein kinase (PKI) comprised more than 90% and type II protein kinase (PKII) less than 10% of the total cAMP stimulated kinase activity. Treatment with 8-CI-cAMP (5  $\mu$ M, 72 h) decreased PKI to a level below 30% of that in untreated control cells and markedly increased PKII composed of three peaks. Photoaffinity labeling/SDS-polyacrylamide gel electrophoresis of column fractions identified the molecular species of regulatory (R) subunits present in protein kinases. Control cells contained high levels of the 48-kDa protein (RI) that composed PKI and low levels of the 50-kDa RII associated with PKII. 8-CI-cAMP treatment brought about a decrease in the 48-kDa RI along with an increased formation of the truncated 34-kDa RI associated with PKI and an increase in the 50-54-kDa species of RII associated with PKII. A similar protein kinase profile as that shown by 8-CI-cAMP treatment was observed in cells infected with the human RII $\beta$  retroviral vector: the 48-kDa RI of PKI decreased and the 52- and 54-kDa RII associated with PKII increased as compared with uninfected control cells. However, unlike 8-CI-cAMP treatment, RII $\beta$  retroviral vector infection brought about no increase in the 34-kDa-truncated RI but exhibited an increase in the free 48-kDa RI subunit. As the 48-kDa RI and the 50-kDa RII were present in control cells, the enhanced expression of the 52- and 54-kDa RII proteins was due to overexpression of the RII $\beta$  gene. We identified the 48-kDa RI as R1 $\alpha$ , the 50-kDa RII as R11 $\alpha$ , the 52-kDa RII as R11 $\beta$ , and the 54-kDa RII as the phosphorylated form of either the R11 $\alpha$  or R11 $\beta$  subunit. *In vivo* labeling experiments using [ $^3$ H]8-CI-cAMP demonstrated that 8-CI-cAMP enters cells and binds to both PKI and PKII. The [ $^3$ H]8-CI-cAMP binding profile of HPLC showed that 8-CI-cAMP selectively down-regulates PKI and up-regulates PKII, and such an effect of 8-CI-cAMP is mimicked by exposing cells to R1 $\alpha$  antisense oligodeoxynucleotide, which suppresses R1 $\alpha$  and enhances R11 $\beta$  expression. Dot-blot hybridization analysis demonstrated that both 8-CI-cAMP treatment (5  $\mu$ M, 6 h) and R11 $\beta$  retroviral vector infection elicited a marked induction of R11 $\beta$  and C $\alpha$  mRNA with little or no change in R1 $\alpha$  and R11 $\alpha$  mRNA. These results suggest that the R1 $\alpha$  and R11 $\alpha$  regulatory subunits determine the distinct roles of type I and type II cAMP-dependent protein kinase isozymes in the regulation of cell proliferation in HL-60 cells and that the formation of the 34-kDa-truncated RI is an efficient mechanism for type I protein kinase down-regulation.

8-CI-cAMP, a site-selective cAMP analog as a novel agent that inhibits the promoter activity of multidrug-resistance (MDR-1) gene. We have shown previously that 8-CI-cAMP inhibits the growth of multidrug resistant (MDR) cell lines and down-regulates p-glycoprotein. In this study, we demonstrate that 8-CI-cAMP inhibits the MDR promoter activity. Our human MDR1 promoter CAT construction, MDRCAT, contains 4.7 kb of a genomic MDR1 sequence upstream of the start of transcription linked to the bacterial CAT reporter gene. MCF-7 cells were transfected with the MDRCAT gene, and the MDR1 promoter activity was measured by CAT expression in cells untreated or treated with 8-CI-cAMP. 8-CI-cAMP treatment (2.5 $\mu$ M) brought about 50 and 90% reduction, respectively, in CAT activity after treatment for 4 and 24 h, as compared to untreated control cells. A metabolite, 8-CI-adenosine, did not mimic the effect of 8-CI-cAMP, indicating that the CAT activity inhibition was due to the intact molecule of 8-CI-cAMP, rather than due to its metabolites. Since the mechanism of 8-CI-cAMP action involves upregulation and nuclear translocation of type II regulatory (RII $\beta$ ) subunit of cAMP-dependent protein kinase, we examined the effect of RII $\beta$  gene by co-transfecting it with MDRCAT gene. It was found that RII $\beta$  gene inhibits the CAT activity mimicking the effect of 8-CI-cAMP. Thus, RII $\beta$  may be the negative regulator of MDR promoter.

Point mutation of type I and type II regulatory subunits (RI versus RII) of cAMP-dependent protein kinase (PKA) by site-directed mutagenesis in the structure-function analysis of R subunits of PKA in growth control. We have previously shown by the use of antisense strategy that the type I regulatory subunit (RI $\alpha$ ) of PKA is a growth stimulatory protein, while the type II regulatory subunit (RII $\beta$ ) is a growth inhibitory and differentiation-inducing protein. The RI and RII subunits can be distinguished by their autophosphorylation and nuclear translocation properties. RII has an autophosphorylation site at a proteolytically sensitive hinge region, while RI has a pseudo-phosphorylation site. The RII but not RI contains a nuclear location signal, KK RK. In order to determine the functional significance of these sequences, the serine<sup>114</sup> at the hinge region of human RII $\beta$  was replaced with an alanine (RII $\beta$  p mut) or the arg<sup>264</sup> of KK RK was replaced with a methionine (RII $\beta$  k mut). Northern blot analysis showed that *Ki-ras*-transformed NIH/3T3 clone DT overexpressing either RII $\beta$  p mut or RII $\beta$  k mut brought about an altered RI $\alpha$  mRNA pattern which was not detected in DT cells overexpressing the wild type RII $\beta$ . These results suggest that both autophosphorylation and nuclear translocation of RII $\beta$  are involved in the regulation of RI $\alpha$  and thus in the growth control of DT cells.

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

PROJECT NUMBER  
 ZO1 CB 04848-21 LTIB

PERIOD COVERED

October 1, 1992 to September 30, 1993

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)

PI: Mary Lou Cutler	Expert	LTIB, DCBDC, NCI
Others: Toshitaka Tsuda	Visiting Fellow	LTIB, DCBDC, NCI
Laura Masuelli	Visiting Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)

Dr. M. Noda, Japanese Foundation for Cancer Research, Tokyo, Japan; Dr. S. Halegoua, S.U.N.Y. at Stony Brook, NY.

LAB/BRANCH Laboratory of Tumor Immunology and Biology

SECTION Office of The Chief

INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 2.3	PROFESSIONAL: 2.3	OTHER: 0
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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		
<input type="checkbox"/> (a2) Interviews		B

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

We have constructed a cDNA library from a *ras* revertant cell line in a eukaryotic expression vector and screened this library for cDNA molecules capable of suppressing *ras* transformation. The screening was accomplished by transfection of the cDNA library into a *ras* transformed cell line and selection for drug resistance and phenotypic change. More than 100 morphologically nontransformed colonies were isolated using this strategy. Two cDNAs isolated from primary transfectants have been found on secondary screening to be capable of suppressing the *ras* transformed phenotype. The first of these cDNA encodes a small RNA, 4.5S RNA, a molecule which is capable of suppressing the *ras* transformed phenotype when it is expressed at a high level. High levels of 4.5S RNA are found in *ras* revertant cell lines and reduced levels in *ras* transformed cell lines compared to the level of this RNA in normal rodent fibroblasts. In addition, another recovered cDNA, referred to as *rsp-1*, is a novel gene which specifically suppresses v-Ki-*ras* and v-Ha-*ras* transformation of fibroblasts and epithelial cells. The *rsp-1* protein contains a series of leucine based repeats homologous to those found in the putative *ras* binding region of yeast adenylyl cyclase. These findings suggest that *rsp-1* may physically associate with *ras* p21 and disrupt Ras signal transduction in this way. *rsp-1* is a phylogenetically highly conserved molecule; cloning and sequencing of the human *rsp-1* cDNA revealed that the human *rsp-1* protein is 96% homologous to the mouse *rsp-1*. Screening of over 100 cell lines and tissue, both human and rodent, revealed that *rsp-1* RNA expression is ubiquitous. The human *rsp-1* gene has been localized to human chromosome 10. Our current efforts are aimed at elucidating the mechanisms by which this molecule disrupts *ras* signal transduction in vitro and determining if the *rsp-1* locus is disrupted in human tumors.

## Major Findings

The major goal of this project is to determine the role of negative regulation in cellular growth and transformation. Specifically, we have sought to identify genes by expression cloning which can suppress *ras* transformation and to characterize the mechanisms and pathways necessary for their phenotypic expression. To identify such genes a cDNA library was constructed in a eukaryotic expression vector using RNA from a *ras* revertant cell line and transfected into the *ras* transformed cell line, DT. Following selection for cells which had taken up cDNA, phenotypically "flat" primary transfectants were isolated. The cDNAs recovered from these transfectants were assayed by a secondary round of screening for *ras* suppressor activity on DT cells. With this procedure more than 100 primary transfectants have been isolated and expanded into cell lines. cDNA have been recovered from more than 20 of these cell lines and tested in a secondary screening assay. Two cDNAs which suppress the *v-ras* transformed phenotype have been identified and characterized.

DNA sequence analysis of the first recovered cDNA with *ras* suppressor activity revealed that it was a clone of 4.5S small nuclear RNA. We have determined that this RNA is expressed at a reduced level in *ras* transformed cells. In addition, it is present at a 5-10 fold higher level in cells resistant to *ras* infection, i.e. *ras* revertants, than in normal cells. The increase in the 4.5S RNA level in the revertant cell lines was attributable to both transcriptional and post-transcriptional events while the reduction in the transformed cells was entirely the result of a decrease in the rate of transcription. Analysis of the level of other small nuclear RNAs in the transformed and revertant cell lines revealed that the mouse B2 repeat sequences share this unusual transcription pattern. Introduction of the gene for 4.5S RNA containing its own promoter elements into a retroviral "double copy" vector allowed us to transfect and infect *ras* transformed cells thereby increasing the level of 4.5S RNA in the recipient cells. Using this methodology it has been possible to suppress the growth of *ras* transformed cells in soft agar, but no stable revertant cell lines have been isolated. 4.5S RNA was characterized as a RNA molecule found associated with preparations of polyadenylated RNA and a role for this small RNA in transport of mRNA has been proposed. The recent discovery that one of the Ras GTPase (*Ras* GAP) associated proteins, p62, is an RNA binding protein suggests that regulation of mRNA may be a function of a *ras* signaling pathway. Our current efforts are aimed at determining whether 4.5S RNA is involved in a regulatory pathway with p62 and what the contribution of this small RNA is to maintenance of the nontransformed phenotype.

Another of the recovered clones is a novel gene referred to as *rsp-1*. The introduction of *rsp-1* cDNA into DT cells suppressed the growth of that cell line in agar by 30-75% and yielded phenotypically flat revertants. *rsp-1* also suppressed anchorage independent growth of a *ras* transformed mouse mammary epithelial cell line suggesting that its effects are not limited to fibroblasts. In addition, a NIH-3T3 cell line containing a copy of the *rsp-1* cDNA under the control of a mouse metallothionein promoter was specifically resistant to retransformation by *v-Ha-ras* and *v-Ki-ras* but not by *v-mos*, *v-src*, or *v-raf*. The *rsp-1* cDNA is not related to any sequences in the nucleic acid data bases. It encodes a 277 amino acid, 33kD protein, the amino terminal two-thirds of which share homology at the amino acid level with the regulatory region of yeast adenyl cyclase. The homology is confined to a series of leucine based repeats 23 amino acids in length which are necessary for the activation of adenyl cyclase by Ras in *Sa. cerevisiae*. This homology, in conjunction with the *ras* suppressor activity, suggests that *rsp-1* may associate with Ras p21 and that its suppressor activity may be a result of this property. Interaction with a highly conserved protein like *ras* would require that *rsp-1* also be phylogenetically highly conserved. Cloning and sequencing the human *rsp-1* cDNA revealed that it too encodes a 277 amino acid protein which is 96% homologous to the mouse *rsp-1*. Western blotting and immunoprecipitation using anti *rsp-1* antibody detect a 33kd protein in human as well as rodent cells. Southern blotting revealed that *rsp-1* is a single copy gene, and that human and mouse *rsp-1* probes hybridize readily at high stringency to DNA from human, monkey, rodent, canine, bovine, feline, avian and xenopus species. In addition, screening of over 100 cell lines and tissues of

rodent and human origin indicated that all contained *rsp-1* RNA. We have localized the *rsp-1* locus to human chromosome 10 and identified a human tumor cell line containing low levels of *rsp-1* RNA and protein. Our current studies are aimed at expanding our knowledge of the role *rsp-1* plays in the regulation of *ras* signal transduction and determining if it can serve as a loss of function mutation activating *ras* signal transduction pathways.

*rsp-1* is rapidly phosphorylated in response to NGF and EGF, indicating it may play a role in signaling from extracellular receptors. *Ras* is an important intermediate on these pathways. Studies of *rsp-1* and its interaction with proteins involved in the *ras* signal transduction pathway will continue in tissue culture assays. In addition, the isolation and identification of proteins binding to or associating with *rsp-1* will proceed *in vitro* using immobilized recombinant *rsp-1* protein. By introducing plasmids encoding truncated, deleted, or mutated versions of *rsp-1* we hope to determine the elements of the protein important for *ras* suppression. We plan to screen samples from human tumors reported to contain alterations involving the *rsp-1* region of chromosome 10; this should allow us to determine if *rsp-1* plays a role in human neoplastic disease. *rsp-1* expression plasmids will be introduced into human tumor cell lines containing low endogenous levels of *rsp-1* protein and these reconstructed cells will be tested for reduction in anchorage independent growth and reduction in ability to form tumors in nude mice. In addition, screening cDNA from the expression cloning assay will continue. We will recover cDNA from our remaining primary transfectants, test them in *ras* suppression assays, and sequence portions of the molecules using an automated sequencing system. This will allow rapid determination of potential homologies to molecules involved in signal transduction and allow conclusions to be drawn concerning the nature of encoded proteins.

### **Publications**

Cutler ML, Bassin RL, Zanoni L, Talbot N. Isolation of *rsp-1*, a novel cDNA capable of suppressing v-*ras* transformation. *Molecular and Cellular Biology* 12;3750-3756. 1992.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08226-17 LTIB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

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

Hormones, Antihormones and Growth Factors in Mammary Development and Tumorigenesis

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

PI:	Barbara K. Vonderhaar	Research Chemist	LTIB, DCBDC, NCI
Others:	Rina Banerjee	Visiting Associate	LTIB, DCBDC, NCI
	Mario Ikeda	Visiting Fellow	LTIB, DCBDC, NCI
	Barbara Terry-Koroma	IRTA Fellow	LTIB, DCBDC, NCI
	Erika Ginsburg	Biologist	LTIB, DCBDC, NCI

## COOPERATING UNITS (if any)

Dr. Sandra Haslam, Michigan State University, East Lansing, MI; Dr. Karen Plaut, University of Vermont, Burlington, VT; Dr. James Zwiebel, Georgetown University, Washington, D.C.; Dr. Frederick Moolten, VA Medical Center, Bedford, MA; Dr. Mary Lou Cutler, LTIB, NCI

## LAB/BRANCH

Laboratory of Tumor Immunology and Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF 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 mammary gland is a complex organ whose growth and development are 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 (PrI), estrogens, and progesterone, with recent work also examining how epidermal growth factor (EGF), and transforming growth factors  $\alpha$  and  $\beta$  are affected by the interplay of these three classical hormones. 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, PrI and glucocorticoids, EGF or TGF- $\alpha$  can promote full lobulo-alveolar development *in vitro*. High concentrations of the somatogenic hormone, growth hormone, can substitute for PrI in this system. This effect is not mediated through insulin-like growth factor I. Lobulo-alveolar development *in vitro* is not inhibited by TGF- $\beta$ ; casein synthesis is. In addition, TGF- $\beta$  dramatically inhibits ductal outgrowth by epithelial cells transduced with the human TGF- $\beta$ 1 gene and transplanted back into the cleared mammary fat pad. PrI induced growth of the mouse mammary epithelial cell, NOG-8, involves activation of protein kinase C (PKC). PrI induces translocation of the PKC from cytosol to the membranes within 10 min. of exposure to the hormone. When NOG-8 cells are transformed with *ras* oncogene they lose the ability to bind PrI when grown in the presence of charcoal stripped serum. This effect is reversed by the addition of PrI to the culture medium, or by introducing the *rsp-1 ras* suppressor gene. Finally, we have explored the relationship of a membrane associated anti-lactogen binding site (ALBS) to the PrI receptor on human breast cancer cell growth in culture. PrI induced growth of human breast cancer cells can be blocked by non-steroidal antiestrogens such as tamoxifen. This action is through the ALBS which appears to be intimately associated with the PrI receptor. These studies are greatly aided by use of monoclonal antibody B6.2 which is the first known such antibody to recognize the human PrI receptor. The antiprolactin action of tamoxifen, working through the ALBS, may have important clinical implications.

### Major Findings

In addition to the classical hormones such as insulin (I), corticoids (A and H), prolactin (PrI), estrogen (E) and progesterone (P), 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.

Glandular development occurs *in vitro* only after priming with estrogen and progesterone (E/P). This priming initiates DNA synthesis *in vivo*, increases EGF binding and decreases the production of TGF- $\beta$  mRNA. E or P alone is not sufficient during the priming process. The addition of E and P to the culture medium cannot overcome the need for priming *in vivo*. Effects on DNA synthesis and EGF receptors are primarily due to P. E increases TGF- $\alpha$  in the gland. 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.

Full lobulo-alveolar development of the mammary gland as well as casein synthesis *in vitro* requires the presence of PrI in the medium. At high concentrations, growth hormone (GH) replaced PrI for lobulo-alveolar development as well as casein gene expression. Addition of EGF to the cultures with either PrI or GH resulted in maximal development. Insulin-like growth factor I did not substitute for PrI or GH nor did it substitute for insulin in tissue maintenance even in the presence of EGF.

Previously we had shown that local, *in vivo*, induction of lobulo-alveolar development occurs in mouse mammary glands exposed to EGF or TGF- $\alpha$  via a cholesterol based pellet inserted directly into the gland. We now show that the cholesterol pellet itself was involved in this reaction. Specific binding of lactogenic hormones to the mammary gland membranes was increased by about 5 fold by the presence of the cholesterol pellet, while the specific binding of EGF was increased 2 fold. These effects of cholesterol were local and direct.

Since TGF- $\beta$ 1 is not involved in lobulo-alveolar development but does play a critical role in the development of the architecture of the mammary gland during puberty, we used the mammary transplant system to investigate its effects on very early development of the gland. For these studies we used a retroviral vector which expresses the human TGF- $\beta$ 1 gene under the control of an internal, inducible, mouse metallothionein-1 (MT) promoter and the neomycin (G418) resistance gene within the first open reading frame of the simian virus 40 promoter. Epithelial cells in primary cultures were infected after stimulation of the cultures with EGF.

After selection of the cells with G418, just prior to harvest for implantation into the cleared fat pad, some cells were exposed to CdCl<sub>2</sub> and induction of the gene confirmed by Southern analysis of PCR products. We transplanted the remaining infected cells into the cleared #4 abdominal fat pads of syngeneic mice and placed the mice on ZnSO<sub>4</sub> in their drinking water. After 6 weeks we found that glands transplanted with freshly isolated mammary epithelial cells or primary cells infected with control vector had normal outgrowths of branching ducts with alveolar buds. However, all outgrowths expressing the MT-TGF- $\beta$ 1 construct had truncated or no ducts and abnormal, enlarged terminal end buds.

PrI-induced growth of the normal mouse mammary NOG-8 cells involves activation of protein kinase C (PKC). Treatment of cells with either PrI or the phorbol ester, PMA, or both together, gave an 8 fold increase in PKC activity with maximal stimulation occurring within 10 minutes of PrI treatment. H7, a potent PKC inhibitor, completely inhibited the hormone induced enzyme activity. Subsequent increases in cell numbers were also effectively blocked by H7. PrI treatment translocated the PKC activity from the cytosol to the plasma membranes.

NOG-8 cells binds PrI when grown in media supplemented with charcoal stripped serum (CSS). However, when these cells were transformed by either point mutated v-Ha-Ras (SR1 cells) or v-Ki-Ras (NOG8NP4 cells), but not *neu*, they rapidly lost the ability to bind lactogens when grown in CSS. PrI binding was restored by returning the cells to FCS or by supplementing the CSS with PrI. Both message for the receptor and receptor protein were present even in cells grown with

CSS. The loss of Prl binding was not due to high levels of TGF- $\alpha$  produced by *ras* transformed cells. When the NOG8NP4 cells were stably transfected with the *ras* suppressor, *rsp-1*, under the control of the metallothionein promoter, they regained normal lactogen binding properties when grown in the presence of CdCl<sub>2</sub> and CSS. In addition, Prl binding to NOG-8 cells grown in CSS or SR1 cells grown in either FCS or CSS plus Prl was inhibited in a concentration dependent manner in the presence of pertussis toxin. Thus, reversion to the parental phenotype by the *ras* suppressor, *rsp-1*, and the suppressive effects of pertussis toxin, suggest that the effect on lactogen binding is a direct one involving the p21<sup>ras</sup> protein product of the *ras* gene.

Prl-induced growth of the human breast cancer cells is inhibited by tamoxifen (TAM) and related non-steroidal, triphenylethylene antiestrogens acting through the membrane associated ALBS. Antiestrogens of the class which bind to the ALBS also inhibit the binding of Prl to its receptor. In studies to characterize the relationship of the ALBS to the Prl receptor we found that TAM acted by inhibiting the binding of Prl to the receptor rather than promoting dissociation of the hormone-receptor complex. In the presence of TAM the affinity of lactogens for the Prl receptor decreased 10-fold. Binding of <sup>3</sup>H-TAM to mammary gland membranes was effectively inhibited by anti-Prl receptor antibody. Isolation and cross-reisolation of Prl receptors and the ALBS by affinity chromatography resulted in co-elution of both binding activities. The isolates from the affinity resins primarily contained a single 90kDa band which was precipitated with the anti-Prl receptor antibody. Lineweaver-Burk analysis suggested that TAM is a competitive inhibitor of the hormone binding. Taken together these data suggest that the ALBS may be one form of the Prl receptor and that TAM and the lactogenic hormones may share a common binding site.

The relationship of the Prl receptor and ALBS to the antigen recognized by the monoclonal antibody B6.2 was examined on T47D cells. Monoclonal antibody B6.2 is an IgG<sub>1</sub> raised against a membrane-enriched fraction from metastatic human breast cancer cells. B6.2 was as effective as polyclonal anti-Prl receptor antibody in inhibiting the binding of Prl to membranes from human tissue and cells. Epidermal growth factor receptors on T47D cells were unaffected by B6.2. Prl induced growth of the T47D cells was blocked by B6.2. Specific binding of B6.2 to the cells was completely inhibited by Prl. Binding of both Prl and B6.2 was inhibited by growing the T47D cells in the presence of tunicamycin A<sub>1</sub>. An affinity column of B6.2 was used to purify a 90kDa protein which specifically bound lactogenic hormones and was precipitated by the polyclonal anti-Prl receptor antibody. Products of tryptic and V8 digests of the B6.2 antigen and purified Prl receptors were identical. Thus these data suggest that the monoclonal antibody B6.2 is an anti-human Prl receptor antibody. This was confirmed by immunocytochemistry showing that mouse 3T3 cells, when stably transfected with the gene for the long form of the human Prl receptor, reacted with B6.2 and polyclonal anti-Prl receptor antibody. Parental 3T3 cells, devoid of Prl receptors, were negative for all antibodies tested. Thus, MAb B6.2, is the first anti-human Prl receptor monoclonal antibody and should provide a useful tool for further studies on purification and characterization of these receptors from human tissues.

**Future Work:** The individual roles of E and P in the priming process will continue to be examined with particular emphasis on induction of the EGF, E and P receptors, and release of the inhibitory effects of TGF- $\beta$ . We will use the transplantation system to examine the effects of TGF- $\beta$ <sub>2</sub> and TGF- $\beta$ <sub>3</sub> on mammary cell outgrowth. Histological examination of the bulbous end buds which occur under the influence of TGF- $\beta$ <sub>1</sub> will be performed in concert with *in situ* hybridization and immunocytochemistry to localize the growth factor. We will continue to characterize the Prl receptor and the ALBS isolated by affinity chromatography and immunopurification and determine what, if any, physical relationship exists between these two molecules. Other Prl responsive, TAM sensitive cell lines will be sought from a variety of human breast, colon, brain and prostate cell lines. *In vivo* studies on inhibition of Prl-induced growth of tumors by TAM will begin.

**Publications**

Vonderhaar BK, Plaut K. Interdependence of hormones and growth factors in lobulo alveolar development of the mammary gland and in tumorigenesis. In: Breast Cancer: Biological and Clinical Progress Ed: L. Dogliotti, A. Sapino, G. Bussolati. Kluwer Academic Publishers, Boston, MA. 1992, pp. 59-80.

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Banerjee R, Ginsburg E, Vonderhaar BK. Characterization of a monoclonal antibody against human prolactin receptors. Int. J. Cancer, in press 1993.





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

PROJECT NUMBER  
 Z01 CB 08907-10 OD

PERIOD COVERED  
 October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Regulation of Immune Response to Tumor Cells

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

PI:	C.-C. Ting	Senior Investigator	OD DCBDC NCI
Others:	M.E. Hargrove	Microbiologist	OD DCBDC NCI
	J. Wand	Visiting Associate	OD DCBDC NCI

COOPERATING UNITS (if any)

Dr. Shu-Mei Liang and Dr. Chi-Ming Liang from the Center for Biologics Evaluation and Research, FDA

LAB/BRANCH  
 Office of the Director

SECTION

INSTITUTE AND LOCATION  
 NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:  
 2.0

PROFESSIONAL:  
 2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

- Protein kinase C and cytokine regulation of CD3-AK-response induced by  $\alpha$ CD3:  
 The CD3-AK model is used to determine the biological function of PKC isozymes in T cell activation. After screening the effect of a panel of different PKC antagonists on the  $\alpha$ CD3-induced proliferation (PR) and cytotoxic response (PR), they were shown to give differential effect on the PR and CR, suggesting that different PKC isozymes are inhibited by these different antagonists. To further determine the production of PKC isozymes by western blot and the expression of PKC mRNA by northern blot will allow us to determine which isozyme(s) is responsible for regulating proliferation or generation of killer cells.
- Glutathione regulation of IL-4 dependent activated killer cells: Glutathione (GSH) regulates the activation and differentiation of IL-4 dependent CD3-AK cells. BSO, a GSH synthetase inhibitor, decreases both the proliferation and cytolytic activity of the killer cells. BSO does not affect the production and exocytosis of the cytolytic granules which mediate the lytic reactions. These results suggest that the reduction of intracellular GSH which regulates the thiol enzyme activity will impair the ubiquitination pathway whose function is critically dependent on thiol enzymes. Thus reduced cellular GSH leads to disruption of normal cell cycle and production of defective cytolytic granules, which results in reduced proliferation and reduced killer cell activity.
- Tumor cell-induced immunosuppression: Tumor cell/cell free products suppress CD3-AK response. IL-4 or PKC antagonists alone partially restores the response and the combination of the two may fully restore the response. These studies should help to devise a strategy to override the tumor-induced immunosuppression.

## Major Findings:

1. Role of protein kinase C and cytokines on the function and production of cytolytic granules in  $\alpha$ CD3-activated killer cell-mediated killing of tumor cells: The effect of PMA and staurosporine (PKC depletor/antagonist) and IL-2/IL4 were used to determine the role of PKC and cytokine on the  $\alpha$ CD3-induced activated killer cells (CD3-AK). The production of BLT-esterase generally correlated with the cytolytic activity of CD3-AK cells and was reduced by PKC depletor/inhibitor but increased by IL-4. In studying the effector function of CD3-AK cells, it was found that adding PMA or SSP at the effector phase inhibited the PKC-dependent slow lysis. PMA but not SSP also reduced fast lysis, which was shown to be an PKC independent event. Additional experiments were performed to determine the effect of PKC on the lytic granules and to determine whether PMA may have other effects on the effector-to-target relationship unrelated to PKC. It was found that neither PMA nor SSP affects the function of cytolytic granules as measured by hemolytic assay against anucleated target (SRBC). These findings indicate that PKC has no direct effect on the granules. When testing against nucleated tumor target with a novel approach using noncytolytic surrogate killers, the lytic activity of the granules was inhibited by PMA, suggesting that exocytosis or delivery of granules to nucleated target cells may require mobilization of intracellular  $Ca^{2+}$  in the killer cells and this process is inhibited by PMA. These findings indicate that PKC and cytokines regulate the production but not the lytic activity of cytolytic granules. Nonetheless, delivery of cytolytic granules from killer cells to the nucleated tumor target appears to be an  $Ca^{2+}$  dependent event unrelated to PKC.

2. Regulation by glutathione of the activation and differentiation of IL-4 dependent activated killer cells: Glutathione (GSH) was shown to regulate the generation of IL-2-dependent activated killer cells. Generation of  $\alpha$ CD3-activated killer cells CD3-AK was regulated by both IL-2 and IL-4. After initial activation of mouse splenocytes by  $\alpha$ CD3, subculturing the CD3-AK cells in IL-4 resulting in the production of IL-4-dependent killer cells whose proliferative and cytolytic activities were abrogated by  $\alpha$ IL-4 antibody 11B11. Adding graded doses of BSO, a GSH synthetase inhibitor, into CD3-AK cells culturing in IL-4 resulted in the reduction of their proliferative and cytotoxic responses. Adding exogenous GSH reversed the inhibitory effect of BSO and restored the proliferation and cytolytic activity of IL-4-dependent CD3-AK cells. The dose requirement for BSO to affect the IL-4-dependent CD3-AK cells was similar to the IL-2-dependent CD3-AK cells. These findings indicate that GSH also regulates the function of IL-4 in the activation and differentiation of CD3-AK cells. To further study the mechanism for the GSH regulation of the cytolytic activity of CD3-AK cells, we found that BSO did not reduce the production of BLT-esterase which contained mostly the cytolytic granules; in fact, BLT-esterase production was often increased by BSO. Furthermore, the exocytosis and effector function of cytolytic granules were also not affected by BSO. Thus it appears that reduction of cellular GSH results in the accumulation of defective cytolytic granules which accounts for the reduction of killer cell cytolytic activity.

3. Function of different PKC isozymes in the regulation of T cell activation: PKC is one of the few biological reagents whose structure has been well

characterized but whose function is poorly understood. Recent molecular cloning and biochemical experiments have led to two major findings. One is that PKC constitutes a family of protein (conventional cPKCs; PKC  $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ) with similar structural and enzymatic properties that distributes in tissues and cells in a type-specific manner. The second finding is that there exists a family of PKC-related proteins (novel PKCs; nPKC  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ) which is clearly different from that of conventional PKCs. The function of these different PKCs is largely unknown. Until now, no agent(s) has been identified to specifically affect the function of a particular isoform of PKC. Transfection of mouse fibroblasts with different PKC genes has offered a powerful tool to analyze this important question, but the study has been largely limited to the subject of cell proliferation. Their effect on T cell activation and differentiation remains to be determined. In our study, it was found that PMA or SSP had selective effect on the proliferative and cytotoxic responses. If both are PKC-regulated phenomenon, then this differential effect may be related to the selective inhibition of different PKC isozymes. Based on this assumption, we started a painstaking process to screen different PKC antagonists and to determine their effect on the CD3-AK response. The experiments are carried out in three phases.

**Phase 1: To determine the differential effect of PKC antagonists on CD3-AK response.**

**Phase 2: Correlation with cytokine and BLT-esterse production.**

**Phase 3: Correlation with the expression of PKC genes and isozyme levels.**

We have already completed phase 1 work. Different PKC antagonists are tested for their effect on the proliferative response (PR) and cytotoxic response (CR) to  $\alpha$ CD3 activation. The rationale is that if these reagents give different effects on PR and CR, then there is a distinct possibility that different PKC isozymes are affected, directly or indirectly. Cell survival is a major problem in working with PKC antagonists in long term cultures. It should be noted that except PMA, using high doses of PKC antagonists will induce cell death. Therefore experiments performed with these reagents have to be done in a very narrow dose range to keep the cells viable and functional during the course of study. After initial screening, the effect of the PKC antagonists can be classified into five categories.

- a. PR decreased, CR unaffected: cardiotoxin, melittin.
- b. PR unaffected, CR increased: 9-aminoacridine, polymyxin B.
- c. PR unaffected, CR decreased: amiloride, staurosporine.
- d. PR increased, CR decreased: PMA.
- e. PR increased, CR increased: mastoparan, sphingosine.

Most of the reagents we tested fall into group a; however, several reagents show differential effect on PR and CR (groups b-e). We are very encouraged by the results. These findings are highly suggestive that different PKC isozymes may be affected by different PKC antagonists. It should be kept in mind that the above phenomenon may be mediated by other effects exerted by these reagents which are unrelated to PKC.

After completion of the screening process, we shall proceed to phase 2 to determine the effect of these reagents on the production of cytokines, i.e., IL-2, IL-4, and TNF., and the production of BLT-esterase. Direct assaying the cytolytic granules by conventional method may be technically difficult, but using a more sensitive granule-surrogate killer assay may help to solve the problem. The IL-2 production in CD3-AK cells is found not to be affected by these different antagonists used at non-toxic doses.

4. Tumor cell-induced immunosuppression and reversal of suppression: Tumor cell and cell free components are found to suppress  $\alpha$ CD3-induced CD3-AK response, both in proliferation and in the generation of killer cells. Tumor cells can activate suppressor mechanism to override host immunity. If these suppressor mechanisms are regulated by PKC, then using PKC agonist/antagonists may augment or inhibit the tumor-induced suppression. In our initial experiments, we found that adding IL-4 or PKC antagonists alone partially restored the CD3-AK response in the presence of tumor cells, and the combination of the two might fully restore the proliferative and cytolytic activity of the CD3-AK cells.

#### Proposed Course of Study:

1. To finish the project on studying the PKC regulation of gene (perforin) expression and protein (BLT-esterase) production in CD3-AK cells.

2. To continue the phase 3 study of regulation by PKC isozymes on T cell activation - correlation with the expression of PKC genes and isozyme levels. This part of the work is pending upon the availability of resources to perform western blot and northern blot analysis.

3. To continue the study on tumor cell-induced immunosuppression and reversal of suppression. Works will be focused on studying the possibility of restoration of immune capacity by cytokine (IL-4) and PKC antagonists/agonists treatment, and their implication in the immunotherapy of tumor growth.

4. To develop a surrogate killer-granule assay for measuring granule-mediated lytic reactions against nucleated targets.

5. To perform combined immunotherapy of murine tumor growth with CD3-AK,  $\alpha$ CD3, IL-4, and PKC antagonists.

#### Publications:

Wu J, Shiver J, Hargrove ME, and Ting CC. Role of protein kinase C and cytokines on the function and production of cytolytic granules in  $\alpha$ CD3-activated killer cell-mediated killing of tumor cells. *Int. J. Cancer* 53:973-977, 1993.

Hargrove ME, Wang J, and Ting CC. Regulation by glutathione of the activation and differentiation of IL-4 dependent activated killer cells. *Cellular Immunol.*, (in press).

CONTRACT RESEARCH SUMMARY

Title: Provide Computer Programming Support Services for the Experimental Immunology Branch

Principal Investigator: Lorenzo F. Exposito  
Performing Organization: SYSTEX, Inc.  
City and State: Beltsville, MD

Contract Number: N01-CB-21002  
Starting Date: 09/30/92                      Expiration Date: 09/29/95

Goal: Develop, and maintain during development, the Advanced Flow Cytometry Data System for the Experimental Immunology Branch (EIB). The system shall consist of specialized software which shall provide comprehensive data transfer, data storage and retrieval, data analysis and system management capabilities for flow cytometry instrumentation in the EIB.

Approach: Work is performed based upon specifications in the Statement of Work. Technical briefings are held to review requirements, and the contractor then designs, produces, installs, tests, and documents the required software.

Progress: Completion of migration of EIB flow cytometry experiments from old instrumentation to the new BDIS FACSTAR PLUS. Development, installation testing, de-bugging and modification of initial versions of the Data Transfer Module, Data Storage and Retrieval Module, Data Analysis Module and System Management Module of the Advanced Flow Cytometry Data System. Installed, configured, tested, and modified multiple BETA-TEST versions of the Laboratory Analysis Package (LAP) flow cytometry software under development by DCRT, NIH. Designed, implemented and tested new modules for the Cluster Analysis Package (CAP) software which provide additional list mode data reduction capabilities for flow cytometry data. Implementation of software for automated archiving which utilizes the tape carousel archiving system. Problem solving and interface with field engineers and software manufacturers for multiple hardware breakdowns and multiple software interface problems.

Significance to Cancer Research: The EIB flow cytometry laboratory provides basic research support to more than 50 investigators within the EIB and elsewhere within DCBDC. Work performed under this contract is required in order for the laboratory to utilize new flow cytometry instrumentation in providing this support. Research investigations supported include studies in the areas of: 1) T cell differentiation, activation, and repertoire generation which are important to our understanding of the basis of immune recognition of self versus non-self; 2) cell surface adhesion molecules which are involved in cell homing, trafficking and metastasis; 3) support of clinical investigations involving bone marrow transplantation for therapy of leukemia and lymphoma; and 4) models of immune deficiency.

Project Officer: Susan O. Sharrow  
Program: Immunology Resource  
Technical Review Group: Ad Hoc Technical Review Committee  
FY 93 Funds: \$71,053

D

## CONTRACT RESEARCH SUMMARY

Title: Production of CEA Protein

Principal Investigators:

Terry Mainprize, Ph.D. and Louis Potash, Ph.D.

Performing Organization:

Program Resources, Inc./DynCorp

City and State:

Rockville, MD

Contract Number: N01-CB-21026-01

Starting Date: 9/30/92

Expiration Date: 9/30//93 (Modified)

**Goal:** To produce clinical grade recombinant baculovirus derived carcinoembryonic antigen (CEA) protein that meet all FDA guidelines for patient administration.

**Approach:** PRI will produce CEA protein from a baculovirus-CEA construct supplied by the NCI. The recombinant baculovirus construct will be grown in a substrate cell line approved by the FDA for production of clinical grade reagents. PRI will purify and concentrate the recombinant protein for use as a vaccine. The vaccine must contain the appropriate CEA protein as demonstrated by ELISA assay. The homogeneity of the CEA protein will be confirmed by SDS-PAGE gel analysis and confirmation of the reactive epitope will be demonstrated on Western blot analysis using monoclonal antibody COL-1. PRI will manufacture, purify, vial and perform all necessary testing for FDA approval of the vaccine for patient administration. PRI will assist the NCI in preparation of the IND application, including Drug Master File protocol documentation.

**Progress:** A recombinant baculovirus-CEA construct was supplied by the NCI. PRI obtained the *Spodoptera frugiperda* cell line from the ATCC, adapted the line to serum free media and generated a Master Seed Bank and Master Working Cell Bank. Baculovirus-CEA protein production has begun and CEA expression has been confirmed by Western blot analysis using monoclonal antibody COL-1. Full scale production, purification and quality control testing is in progress. The contract was modified to provide a two month delivery date extension. The clinical grade baculovirus derived CEA protein is expected to be shipped to the NCI drug repository on or before the delivery date.

**Significance to Cancer Research:** This master agreement order will provide clinical grade recombinant baculovirus derived CEA protein for the potential treatment of cancer patients as an anti-cancer vaccine or as a boost using recombinant vaccinia-CEA or avipox-CEA as the primary immunogen.

Project Officer:

Kathleen Siler, M.S.

Program:

Immunology Resource

Technical Review Group:

Cancer Biology-Immunology Contracts  
Review Committee

FY 93 Funds:

\$146,316

D

## CONTRACT RESEARCH SUMMARY

Title: Feral Mouse Breeding Colony

Principal Investigator: Ms. Evelyn Hogg  
Performing Organization: Organon Teknika/Biotechnology Research Institute  
City and State: Rockville, MD

Contract Number: N01-CB-21055

Starting Date: 12/01/91

Expiration Date: 11/30/94

Goal: Induction of mammary tumors with biological (hormones, retroviral shuttle vectors, and mouse mammary tumor virus, MMTV) and chemical carcinogens in various feral strains of *Mus musculus* and other species of *Mus*. Breeding of transgenic strains of *Mus musculus* containing certain activated proto-oncogenes. Maintenance of preneoplastic mammary hyperplastic outgrowth lines by transplantation in syngeneic mice.

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 breeding nucleus. The breeding nucleus is composed of three pedigreed outbred colonies of feral mice having unique characteristics that are pertinent to the study of mouse mammary tumorigenesis. They are: CZECHII V-mice (*Mus musculus musculus*), CZECHII V+, MS (*M. spretus*) mice. Three transgenic mouse lines containing the *Int-3*, *Wnt-1*, and *TGF $\alpha$*  transgenes are also being maintained. These mouse strains are being used to determine the consequences of transgene expression on mammary gland development and mammary tumorigenesis. In addition, a limited breeding nucleus of the high-incidence C3H/OuJ, BALB/cCZECHII, and BALB/cMS inbred mouse strains and the low-incidence BALB/cp and FVB inbred mouse strains are maintained.

Progress: Forty primary hyperplastic mammary outgrowths from CZECHII V+ females have been collected and await analysis. Twelve serial transplantable CZECHII V+ hyperplastic mammary outgrowth lines have been developed and are in their 4-8th passage. DNA analysis of these outgrowths and their tumors have indicated that *Wnt-1* was activated in 2 lines and that these lines are clonally derived. Moreover, all tumors arising within this population possess the identical mutation at *Wnt-1* as well as additional MMTV-induced mutations. Twenty-one independent lung metastases have been collected from one of these *Wnt-1* positive hyperplastic outgrowths. Preliminary studies indicate that each of these contain new MMTV-induced mutations that may be relevant to progression to the metastatic phenotype. We have discovered a new common insertion site for MMTV in another outgrowth line (CZZ1) designated *Int-6*. Preliminary studies indicate that *Int-6* is a unique gene which has been conserved throughout evolution from insects to mammals.

Significance to Cancer Research: Provides essential support for the study of mammary tumorigenesis with the specific goal of identifying and characterizing the genes at risk to MMTV activation. Provides an *in vivo* model to determine the consequences of aberrant oncogene or proto-oncogene expression on mammary gland development and mammary tumorigenesis.

Project Officer: Dr. Robert Callahan

Program: Immunology Resource

Technical Review Group: DEA; Ad Hoc Intramural Technical Review Group

FY 93 Funds: \$114,242

CONTRACT RESEARCH SUMMARY

Title: Induction, Transplantation, and Preservation of Plasma Cell Tumors and Development of Special Mouse Strains

Principle Investigator: Judith Wax  
Performing Organization: Organon Teknika/BRI  
City and State: Rockville, MD

Contract Number: N01-CB-21075  
Starting Date: 02-01-92      Expiration Date: 01-31-97

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, as well as a strict SPF facility for the maintenance of SPF-BALB/cAnPt and BALB/cAnPt nu/nu mice, suitable for maintaining mice for long term plasmacytoma induction experiments. Develop BALB/c congenic strains carrying plasmacytomagenesis resistance (PCT-R) genes. Carry out procedures for identifying markers used in the construction of congenic strains. Maintain colonies of pedigreed wild mice, supplies ascites, tissues, high molecular weight DNA, pedigreed breeders to qualified investigators and collaborators.

Progress: Contractor has carried out basic induction experiments and prepared mice for the various studies carried out in the LG. We have made an effort to curtail long term induction experiments up to 300 days and use more rapid assays such as the focus assay and the new PCR assay. Contractor continues to test transforming viruses in short term experiments. We have introduced a new plasmacytomagenic agent, dimethylpoly-siloxane, currently in the form of a gel and are working out the basic dosimetry and latent periods. This agent induces far less inflammation than pristane. We are currently reconstituting our BALB/c colony with BALB/cAnPt mice after finding that the line used since 1989 has a reduced incidence of plasmacytomas. Contractor's major effort is to produce new congenic and hybrid strains of mice that are being used to find and identify genes that determine susceptibility and resistance to tumor formation. Beginning with congenic strain carrying large segments of DBA/2 chromatin from chromosomes 1,4 and 11, we are developing recombinants that contain more restricted segments. Using inbred SENCAR mice developed on the contract, we are crossing BALB/c (a skin tumor resistant strain) and SENCAR (a highly susceptible strain) to find genes that determine hyperpromotibility.

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 the induction of plasmacytomas. 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 93 Funds: \$947,377 Est. B



## CONTRACT RESEARCH SUMMARY

**Title:** Production of a HTA-Vaccinia Virus Construct

**Principal Investigator:**

Gail Mazzara, Ph.D.

**Performing Organization:**

Therion Biologics Corporation

**City and State:**

Cambridge, MA

**Contract Number:** N01-CB-21154-01

**Starting Date:** 6/30/92

**Expiration Date:** 7/15/93

**Goal:** To construct and produce clinical grade recombinant vaccinia vaccine containing the gene for point mutated ras (glycine→valine) at codon 12 that meet all FDA guidelines for patient administration.

**Approach:** Therion will construct a recombinant ras vaccinia virus containing a point mutated ras gene at codon 12 (glycine→valine). The vaccinia construct will be grown in a substrate that has been approved by the FDA for production of clinical grade reagents. Therion will purify and concentrate the recombinant ras vaccinia vaccine. The vaccine must contain the appropriate cDNA sequence for the inserted gene as demonstrated by Northern and/or Southern blot analysis. The recombinant ras vaccinia vaccine must contain a specific reactive epitope as demonstrated on Western blot analysis using a monoclonal antibody specific for ras. Therion will manufacture, vial and perform all necessary testing for FDA approval of the vaccine for patient administration. Therion will assist the NCI in preparation of the IND application, including Drug Master File documentation.

**Progress:** A plasmid vector was used to generate a recombinant vaccinia virus (vT114) expressing point mutated ras gene (glycine→valine) at codon 12. The point mutated ras gene was supplied by the NCI. The vector also contained the E. coli LacZ gene to allow viral plaque selection using expression of B-galactosidase and point mutated ras in the presence of a histochemical substrate for the enzyme. Following plaque purification, the recombinant ras vaccinia clone was analyzed with respect to genomic structure by Southern blot analysis using ras-specific and vaccinia-specific probes. Ras protein expression was confirmed by Western blot analysis using a ras specific monoclonal antibody. A seed stock of ras recombinant vT114 was used to generate a Master Virus Stock for vaccine manufacture. Manufacture of bulk material was performed. The product has been purified and concentrated by centrifugation. It has been vialated approximately  $10^{10}$  plaque forming units (pfu) per 0.1ml. Quality control testing is in progress and on schedule. The vaccine is expected to be shipped to the NCI drug repository on or before the delivery date.

**Significance to Cancer Research:** The point mutated ras oncogene (position 12 mutation) is expressed to varying degrees on pancreatic, colon, lung, endometrial, thyroid, oral, laryngeal hepatocellular and bile duct carcinoma, as well as melanoma, acute myeloblastic leukemia, basal cell carcinoma and squamous cell carcinoma. This master agreement order will provide clinical grade recombinant ras vaccinia vaccine for the potential treatment of cancer patients.

**Project Officer:**

Kathleen Siler, M.S.

**Program:**

Immunology Resource

**Technical Review Group:**

Cancer Biology-Immunology Contracts  
Review Committee

**FY 93 Funds:**

\$168,731

D

## CONTRACT RESEARCH SUMMARY

**Title:** Production of a HTA-Vaccinia Virus Construct

<b>Principal Investigator:</b>	Gail Mazzara, Ph.D.
<b>Performing Organization:</b>	Therion Biologics Corporation
<b>City and State:</b>	Cambridge, MA
<b>Contract Number:</b> N01-CB-21154-02	
<b>Starting Date:</b> 6/30/92	<b>Expiration Date:</b> 8/30/93

**Goal:** To construct and produce clinical grade recombinant vaccinia vaccine containing the prostate specific antigen gene that meet all FDA guidelines for patient administration.

**Approach:** Therion will construct a recombinant vaccinia virus construct containing the prostate specific antigen (PSA) gene. The vaccinia construct will be grown in a substrate that has been approved by the FDA for production of clinical grade reagents. Therion will purify and concentrate the recombinant vaccinia-PSA vaccine. The vaccine must contain the appropriate cDNA sequence for the inserted gene as demonstrated by Northern and/or Southern blot analysis. The recombinant vaccinia-PSA vaccine must contain a specific reactive epitope as demonstrated on Western blot analysis using a monoclonal antibody specific for PSA. Therion will manufacture, vial and perform all necessary testing for FDA approval of the vaccine for patient administration. Therion will assist the NCI in preparation of the IND application, including Drug Master File documentation.

**Progress:** A plasmid vector was used to generate a recombinant vaccinia virus expressing PSA. The vector contained the PSA gene and the E. coli LacZ gene to allow viral plaque selection using expression of B-galactosidase and PSA expression in the presence of a histochemical substrate for the enzyme. Following plaque purification, the recombinant clone (vT1001) was analyzed with respect to genomic structure by Southern blot analysis using PSA-specific and vaccinia-specific probes. PSA protein expression was confirmed by Western blot analysis using a commercially available rabbit antibody specific for PSA. A seed stock of recombinant vaccinia-PSA virus was used to generate a Master Virus Stock for vaccine manufacture. Manufacture of bulk material was performed. The product has been purified and concentrated by centrifugation. It has been vialled and quality control testing is in progress and on schedule. The vaccine is expected to be shipped to the NCI drug repository on or before the delivery date.

**Significance to Cancer Research:** This master agreement order will provide clinical grade recombinant vaccinia-PSA vaccine for the potential treatment of prostate cancer patients.

<b>Project Officer:</b>	Kathleen Siler, M.S.
<b>Program:</b>	Immunology Resource
<b>Technical Review Group:</b>	Cancer Biology-Immunology Contracts Review Committee
<b>FY 93 Funds:</b>	\$168,731

D

## CONTRACT RESEARCH SUMMARY

**Title:** Production of a HTA-Vaccinia Virus Construct

**Principal Investigator:**

Gail Mazzara, Ph.D.

**Performing Organization:**

Therion Biologics Corporation

**City and State:**

Cambridge, MA

**Contract Number:** N01-CB-21154-03

**Starting Date:** 6/30/92

**Expiration Date:** 10/15/93

**Goal:** To construct and produce clinical grade recombinant vaccinia vaccine containing the gene for point mutated ras (glycine→cysteine) at codon 12 that meet all FDA guidelines for patient administration.

**Approach:** Therion will construct a recombinant ras vaccinia virus containing a point mutated ras gene at codon 12 (glycine→cysteine). The vaccinia construct will be grown in a substrate that has been approved by the FDA for production of clinical grade reagents. Therion will purify and concentrate the recombinant ras vaccinia vaccine. The vaccine must contain the appropriate cDNA sequence for the inserted gene as demonstrated by Northern and/or Southern blot analysis. The recombinant ras vaccinia vaccine must contain a specific reactive epitope as demonstrated on Western blot analysis using a monoclonal antibody specific for ras. Therion will manufacture, vial and perform all necessary testing for FDA approval of the vaccine for patient administration. Therion will assist the NCI in preparation of the IND application, including Drug Master File documentation.

**Progress:** A plasmid vector was used to generate a recombinant vaccinia virus (vT122) expressing point mutated ras gene (glycine→cysteine) at codon 12. The point mutated ras gene was supplied by the NCI. The vector also contained the E. coli LacZ gene to allow viral plaque selection using expression of B-galactosidase and point mutated ras in the presence of a histochemical substrate for the enzyme. Following plaque purification, the recombinant ras vaccinia clone was analyzed with respect to genomic structure by Southern blot analysis using ras-specific and vaccinia-specific probes. Ras protein expression was confirmed by Western blot analysis using a ras specific monoclonal antibody. A seed stock of ras recombinant vT122 was used to generate a Master Virus Stock for vaccine manufacture. Manufacture of bulk material was performed. The product has been purified and concentrated by centrifugation. It has been vial and quality control testing is in progress and on schedule. The vaccine is expected to be shipped to the NCI drug repository on or before the delivery date.

**Significance to Cancer Research:** The point mutated ras oncogene (position 12 mutation) is expressed to varying degrees on pancreatic, colon, lung, endometrial, thyroid, oral, laryngeal hepatocellular and bile duct carcinoma, as well as melanoma, acute myeloblastic leukemia, basal cell carcinoma and squamous cell carcinoma. This master agreement order will provide clinical grade recombinant ras vaccinia vaccine for the potential treatment of cancer patients.

**Project Officer:**

Kathleen Siler, M.S.

**Program:**

Immunology Resource

**Technical Review Group:**

Cancer Biology-Immunology Contracts  
Review Committee

**FY 93 Funds:**

\$168,731

D

## CONTRACT RESEARCH SUMMARY

**Title:** Production of a HTA-Avixox Construct

**Principal Investigator:**

Enzo Paoletti, Ph.D.

**Performing Organization:**

Virogenetics Corporation

**City and State:**

Troy, NY

**Contract Number:** N01-CB-21155-01

**Starting Date:** 8/30/92

**Expiration Date:** 12/7/93

**Goal:** To construct and produce clinical grade recombinant avixox vaccine containing the carcinoembryonic antigen (ALVAC-CEA) that meet all FDA guidelines for patient administration.

**Approach:** Virogenetics will generate a recombinant avixox (ALVAC) construct containing the CEA gene. The ALVAC construct will be produced according to FDA requirements for clinical grade reagents by a subcontractor, Pasteur Merieux, Lyon, France. The vaccine must contain the appropriate cDNA sequence for the inserted gene as demonstrated by Northern and/or Southern blot analysis. The recombinant ALVAC-CEA vaccine must contain a specific reactive epitope as demonstrated on Western blot analysis using monoclonal antibody COL-1, which is specific for CEA. The ALVAC-CEA will be manufactured, vialled and quality control tested to meet FDA approval of the vaccine for patient administration. Virogenetics will assist the NCI in preparation of the IND application, including Drug Master File documentation.

**Progress:** The gene encoding CEA which was supplied to Virogenetics was modified to generate the ALVAC donor plasmid pH6.CEA.C3.2. The ALVAC-CEA construct was produced by homologous recombination. The CEA-containing recombinant was identified by plaque hybridization, plaque purified and amplified. The recombinant has been designated vCP248. The recombinant ALVAC-CEA construct was analyzed with respect to genomic structure by Northern blot analysis and immunoprecipitation. CEA protein expression was confirmed by Western blot analysis using monoclonal antibody COL-1. The recombinant construct has been sent to Pasteur Merieux for generation of the Master Virus Stock (production batch) and for vaccine production. Manufacture of the vaccine is in progress. The ALVAC-CEA vaccine is expected to be shipped to the NCI drug repository on or before the delivery date.

**Significance to Cancer Research:** This master agreement order will provide clinical grade recombinant avixox CEA vaccine for the potential treatment of cancer patients.

**Project Officer:**

Kathleen Siler, M.S.

**Program:**

Immunology Resource

**Technical Review Group:**

Cancer Biology-Immunology Contracts  
Review Committee

**FY 93 Funds:**

\$156,235

D

CONTRACT RESEARCH SUMMARY

Title: Radioimmunoassay and Enzyme Immunoassay of Immunoglobulin Molecules and Antibodies

Principal Investigator: Mr. Norman Beaudry  
Performing Organization: Hazleton Washington, Inc.  
City and State: Vienna, VA

Contract Number: N01-CB-33036 (Successor to Contract N01-CB-71010)  
Starting Date: 06/30/93 Expiration Date: 06/29/97

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 to administered antigens and to murine and human monoclonal antibodies for the study of IL-2 receptor directed therapy of human neoplasia.

Progress: The contractor has established the required radioimmunoassays and the ELISA assays. Elevated IL-2 receptor levels have been demonstrated in the sera of patients with HTLV-1-associated adult T-cell leukemia, HIV-associated AIDS, hairy cell B-cell leukemia or Hodgkin's disease. The assays for murine monoclonal antibodies, as well as human anti-murine antibody responses, has been developed and applied to the study of patients receiving IL-2 receptor directed therapy.

Significance to Cancer Research: These studies help elucidate the abnormalities of the immune system associated with the development of cancer and will assist in the categorization of malignancies of the lymphoid system. Most importantly, 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. The assays for antibodies to murine and human antibodies to the IL-2R $\alpha$  protein are required for the adult T-cell leukemia protocols that involve the use of murine anti-Tac, humanized anti-Tac and yttrium-90 modified anti-Tac.

Project Officer: Dr. Thomas A. Waldmann  
Program: Cancer Biology Resource  
Technical Review Group: DEA; Ad Hoc Intramural Technical Review Group  
FY 93 Funds: \$281,958 (est.)

B

CONTRACT RESEARCH SUMMARY

Title: Maintenance of an Animal Holding Facility and Provision of Associated Research Services

Principal Investigator:  
Performing Organization:  
City and State:

Ms. Leanne DeNenno  
Bioqual, Inc.  
Rockville, MD

Contract Number: N01-CB-85607  
Starting Date: 11/01/87

Expiration Date: 10/31/93

Goal: Maintain colonies of up to about 10,000 inbred mice, 500 inbred rats, 50 hamsters, and 40 rabbits and carry out selected protocols with these animals as specified by the Project Officer. These animals are to be maintained in support of intramural research programs in the Experimental Immunology Branch, DCBDC, NCI.

Approach: Colonies of mice, rats, hamsters, 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 Experimental Immunology Branch. As such, the contractor has maintained colonies of up to about 10,000 mice, 500 rats, 50 hamsters, and 40 rabbits. Breedings of certain strains of mice for experimental needs have been performed when such animals have not been available commercially. Frozen samples of sera and cells, stored in freezers of appropriate temperatures, have been transferred to NIH as required.

Performance on this contract has been very satisfactory. The animal colonies are being maintained according to National Research Council standards. Animal health has, in general, been good. Protocols have been carried out in a satisfactory fashion. Record keeping 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 for support of intramural research programs in the Experimental Immunology Branch, DCBDC, NCI. Many of these programs are concerned with the immune response to cancer.

Project Officer: Dr. Richard J. Hodes  
Program: Immunology Resource  
Technical Review Group: DEA; Ad Hoc Intramural Technical Review Group  
FY 93 Funds: \$873,282

B

CONTRACT RESEARCH SUMMARY

Title: Facility for Preparing and Housing Virus Infected Mice, Genetically Manipulated Mice and Chimeric Mice

Principal Investigator: Ms. Kinta Diven  
Performing Organization: Bioqual, Inc.  
City and State: Rockville, MD

Contract Number: N01-CB-85608  
Starting Date: 09/30/88

Expiration Date: 09/29/93

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, DCBDC, 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, preparations of radiation chimeric mice, thymus transplants and the breeding, care, and manipulation of SCID mice. Protocols and details of experiments are to be carried out as directed by the Project Officer.

Progress: Experiments have been performed that involve irradiation and bone marrow transplantation, thymectomy, immunization, viral preparations bleeding, thymus and skin grafting, and breeding, care and manipulation of SCID mice.

This contract has been recompeted. An award for a four-year successor contract to continue this work will be made October 1, 1993.

Significance to Cancer Research: This experimental mouse facility is required to support the intramural research programs of the Experimental Immunology Branch, DCBDC, 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 related to viral infection, genetic manipulation of hematopoietic reconstitution of the immune system.

Project Officer: Dr. Gene M. Shearer  
Program: Immunology Resource  
Technical Review Group: DEA; Ad Hoc Intramural Technical Review Group  
FY 93 Funds: \$ 0

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